Title of the Invention

Methods for Detecting and Mapping Genes, Mutations and Variant Polynucleotide Sequences

Background of the Invention

DNA and RNA detection is generally accomplished by long DNA probes or by short oligonucleotide probes that complex to the target sequence by complementary hybridization. Long probes consist of DNA copies that range from many hundreds to many thousands of bases in length and are derived from various sources including mRNA, isolated and cloned fragments, or PCR amplification of genomic or cloned DNA. Such probes are usually labeled by nick translation, random priming, PCR or synthesis. They are then hybridized to the target and detected by radioactive, chemiluminescent, colorimetric or fluorescent signaling.

Since signal strength is essentially proportional to the length of the probe, longer DNA probes are sometimes more advantageous. However, long probes have several principal limitations. First of all, specificity is limited since cross hybridization can occur due to similar sequences in gene families, pseudogenes and repetitive DNAs. This problem can be somewhat relieved by blocking DNA technology, but this procedure, e.g., U.S. Pat. Nos. 5,447,871, entails added steps and expense. Hybridization kinetics is also slower, and many hours or even days may be required to effect thorough reassociation of the probe and target sequences even if the probes are fractionated into smaller pieces. It is also more difficult for long probes to penetrate the structural matrix of solid support membranes and fixed tissue. Furthermore, signaling is more efficient in shorter probe lengths in the range of about 500 to about 1500 base pairs since such lengths allow secondary interactions between probe components that multiply the number of probe molecules complexed to each DNA or RNA target site. More importantly, however, unless a sufficiently large piece of target DNA/RNA has been isolated and is in hand to create the probe, large probes cannot be generated since synthetic DNA cannot be efficiently manufactured in lengths greater than 100 base pairs. Consequently, many years have often been expended in simply finding, isolating and copying an adequate portion of the correct DNA target from natural sources.

Conversely, DNA detection can be achieved with short synthetic oligonucleotide probes that usually range in size from 20 to 50 bp in length, and such probes can be manufactured from published sequences. While short oligonucleotides provide a more convenient and rapid route to probe development and use, they have far less signaling capacity since maximal labeling is largely limited to the signal amplification potential of terminal transferase tailing. This procedure allows generation of a small group of 10 to 25 bases attached to the 3' end of the oligonucleotide, resulting in a small number of radioactivity labeled bases or accessible haptenated bases for secondary detection. Consequently, synthetic oligonucleotide probes are mostly used to detect amplified or concentrated target samples. For *in situ* mapping of genomic DNA , they can only be used to detect repetitive sites where sufficient signaling can be achieved by binding multiple copies of the probe to multiple tandem targets.

In related art, Urdea et al. (U.S. Pat. No. 5,124,246) describe a method for amplifying signaling from oligonucleotide probes by sandwiching them to multimers of "comb-like" or linear configurations that are constructed by chemically or enzymatically joining a multiplicity of short synthetic nucleic acids and labeling compounds. These multimers are known as branched or bDNA. However, these multimer structures, which comprise tree or more end-joined parts, depend on the force of hybridization to thether them to the specific probe and the target sequence. Consequently, limited branch structures may be joined to one site and many probes with labeled multimers are required to achieve signaling comparable to long probes. For detecting the N. gonorrhea Tem-1 beta lactamase gene, for example, they describe twenty six oligonucleotide probes that target different sites along the gene, each with an attached multimer.

Separately, Nilsen and Prensky, (U.S. Pat. No. 5,487,973) describe a probe and signaling dendrimer constructed of synthetic nucleic acids that form a layered ball, in which the outer layer consists of free and protruding single stranded oligonucleotide arms of two types. Typically, these dendrimers are custom constructed to target a specific nucleotide sequence, and one type of the multiple free arms will share a specific sequence complementary to the target site, and the other type of the multiple free arms will carry signaling compounds or agents. This design can provide signaling which exceeds that of a

single oligonucleotide probe, but it depends primarily on the limited forces of hybridization provided by one probe arm to tether the sizable dendrimer.

In related art, Newton et al. [Nucleic Acids Research, Vol. 21, No. 5, pp. 1155-1162 (1993)] describe a synthesized DNA probe for solid phase capture or detection by employing primers with phospharamidite spacers. PCR products made with such primers have 5 prime single stranded tails that can bind a target or a capture molecule. Detection of the probes in limited to solid phase capture.

Separately, Nilson et al. [Science, Vol. 265, pp. 2085-2088 (1994)] describe long oligonucleotide probes which have two target specific sequences at each end that separately hybridize to adjacent targeted regions in an end to end manner and are then ligated together. Between the target specific sequences at each end lies a long chain of carbon spacers. Once ligated, these probes encircle the DNA and cannot be removed by denaturation. However, the length of these probes make them difficult to synthesize, and their signaling potential is similar to tailed oligonucleotides since the bases that can be labeled are limited. Consequently, their mapping effectiveness is largely limited to repetitive copy sites.

Somers et al. 1994 [Nucleic Acids Research, Vol. 22, pp. 4840-4841(1994)] describe a method for detecting single base mutations by hybridizing two oligonucleotide probes side by side and ligating them together. One probe has a biotin component which provides a means for capture to an avidin coated microtiter plate. The other probe has a signal hapten digoxigenin which can be conjugated to a colorimetric or chemiluminescent signaling molecule. The Somers' methodology is derived from other published methods on oligonucleotide ligation assays (OLAs). See, e.g. U.S. 4,988,617.

Vo-Dinh et al. 1994 [Anal. Chemistry, Vol. 66, pp. 3379-3383 (1994) and U.S. Pat. No. 5,721,102] describe a method for detecting simple oligonucleotide probes end labeled with a single dye molecule by employing laser light to produce a surface enhanced Raman shift in spectral emissions. This method suggests that other dyes or other labeling agents with a Raman spectral signature can be combined with gene probes to give distinct signaling. However, the signaling potential of this method is limited to one labeling molecule per each probe that is employed.

Applicant has previously devised a simple Gene Amplification Probe (GAP) probe suitable for providing the signal strength of longer DNA probes, but with the specificity and

ease of manufacture of short oligonucleotide probes. Such probes consist of a target specific oligonucleotide attached to a DNA reporter molecule at one end. However, in practice, GAPs are difficult to hybridize effectively and durably when long reporter DNAs of 500 bp length or greater are utilized. Consequently they have produced inconsistent signaling results proportional to reporter tail length, e.g., nylon membrane dot blots. They have also failed to hybridize and signal as effectively as oligonucleotide probes of identical target sequence in *situ* hybridization formats such as sectioned brain, lung and prostate tissues.

To overcome these experimental difficulties, applicant has modified the GAP probe design to give two probes ligated together, known as the GAP-LOCK probe, one of the embodiments of the present invention. In a particular embodiment, the GAP-LOCK probe has ~~a generic linker, known as a probe linker, at one or both distal ends.~~ The probe linker provides for the attachment of one or more reporters, such as the reporter molecules of the present invention. The GAP-LOCK probe was discovered to provide more stable support for long reporters or multiple reporters and it is especially suitable for detecting single base pair changes in a polynucleotide sequence, e.g., mutations and polymorphic variation. The unique advantage of the GAP-LOCK design is its ability to achieve both high specificity and greater signal strength due to its capacity for exponential reporting or labeling.

In another embodiment of the present invention, Applicant has also devised and discovered a WRAP-PROBE, suitable for detecting the presence of a polynucleotide sequence. The WRAP-PROBE has a critical DNA sequence complementary to the target ~~sequences as well as a generic linker, known as probe linkers,~~ at one or both ends. The probe linker provides for the attachment of one or more reporters, such as the reporter molecules of the present invention. The advantage of the WRAP-PROBE is its suitability for exponential reporting or labeling without the need for ligation. This probe also has a simple modular design that allows cheap and easy probe development.

Applicant has also devised and discovered a GENE-TAG reporter method that has amplified signaling when added to a WRAP-PROBE or to other probes hybridized to a target. The GENE-TAG reporter has a generic reporter sequence as well as one or more generic linker ends, known as reporter linkers. The GENE-TAG reporter molecules are designed to provide exponential signaling by virtue of the fact that multiple reporters can be linked to one another end-to-end to form chained or branched structures of such reporter molecules by

hybridization of complementary reporter linkers. Moreover, the generic linkers employed allow these reporter constructs to be joined to a variety of gene probes which contain complementary probe linkers such as the WRAP-PROBEs and GAP-LOCK probes described in the present invention. These GENE-TAG reporters can be prejoined to one another, can be prejoined to the probes, can be joined to the probes after hybridization to the target, and can be self-assembled or layered in situ to form linear or branched arrays that are attached to the probe. The unique advantage of the GENE-TAG method is its suitability for attaching such reporters in a modular manner to probes of different type and to probes specific to different targets. This method is also more suitable for adjusting the degree of amplified labeling according to circumstances.

Moreover, Applicant has a polynucleotide structure, known as a Multi-LINKER, that can be interposed between the probe element and the reporter elements to allow the binding of multiple reporters or reporter chains to probe, such as the WRAP-PROBE of GAP-LOCK probe of the present invention. These Multi-LINKERS can be singular or multiple oligonucleotides made with a series of terminal or internal linking sequences. The composite Multi-LINKER can self-assemble by overlapping complementary linking sequences of two or more oligonucleotides. The Muli-LINKER has a proximal linker end constructed to be joined to a variety of gene probes which contain complementary linkers such as the WRAP-PROBEs or GAP-PROBEs of the present invention and two or more distal linkers to bind multiple reporters. The Multi-LINKER unit can be used in combination with reporters such as short oligonucleotides with labeling units, or with the GENE-TAG reporters of the present invention. The unique advantage of the Multi-LINKER is its ability to attach multiple reporters and the resulting capacity for exponential reporting.

Applicant has also devised a COLOR-TAG modification of the GENE-TAG signal amplification method that is capable of binding or capturing to a probe a specific mix of different GENE-TAG reporters that contain different labeling compounds. This is accomplished by joining a Multi-LINKER structure to the probe that will attract and join GENE-TAGs of a single or mixed color or will join a mix of GENE-TAGs of different colors. This modular design can thereby give each probe a distinct signaling profile based on the mix and or proportion of different labeling compounds which result. This COLOR-TAG method is designed to allow mixing of generic GENE-TAG reporters with complementary

colors, for example, such as red, green and blue colored fluorescent signaling, and thus each probe could achieve a distinct signature color in the same way that red, green and blue light is ratio mixed in television monitors to create a multitude of distinct colors. The unique advantage of this method is that it allows the simultaneous application of multiple probes, with each probe carrying or capturing of GENE-TAG reporters that define and differentiate that probe from other probes.

Applicant has also devised a Capture GAP-LOCK method that is more suitable for detecting small targets or unstable targets like RNA, or where liquid hybridization conditions are needed or more favorable. This design provides a first probe element with a capture molecule or a capture potential and no reporter potential and a second probe element providing reporter potential. Thus, after ligation has joined the first and second probe due to correct alignment on the target, the target can be removed or degraded and effective detection can result due to the fact that the reporter is captured via the ligated probe.

Applicant has also devised RING-TAIL modifications of both the GAP-LOCK and WRAP-PROBE designs that cause these probes to encircle the target polynucleotide upon application of the method. Some of these components are preferably crosslinked together. The combination of a RING-TAIL unit with GAP-LOCK components produces a circular RING-LOCK probe that is closed by ligation. The combination of a RING-TAIL unit with a WRAP-PROBE produces a circular WRAP-LOCK probe that is closed by hybridization, and these hybridized components may then be crosslinked together to form a covalently closed circle. The unique advantage of these RING-TAIL modified methods is that they make the probe highly resistant to removal. The RING-LOCK probe is also particularly suitable for detecting rare mutations by applying double probes with two different colors or labels that separately detect the altered sequence in both the sense and antisense strands of genomic DNA. This enhanced DOUBLE-LOCK method offers the potential to detect pre cancerous cells hidden in a sea of normal cells or to detect mutant virus about to emerge and overwhelm a weakened host.

Summary of the Invention

The invention disclosed herein includes methods, reagents and compositions for detecting nucleic acid sequences, for mapping such sequences in genomic samples or tissues,

and for discriminating polymorphic genetic variations or single base mutations. The invention includes a number of related designs for gene probe components, multilinking components and signaling components, all of which are modular in nature and can be used together or in part. Virtually all of the probe, reporter and multilinking components are constructed of synthetic oligonucleotides or amplified DNAs, and they are generally joined together in composite structures by hybridization of complementary sub-segments, called linkers. Linkers are designed to avoid sequences that occur in the targeted samples and they are designed to facilitate binding by crosslinking or other chemical methods as needed. The reporters are also designed to be conjoined into arrays that can provide amplified signaling. The multilinking components of the present invention may be interposed between the probe and the reporter units and provide for the binding of multiple reporters. Various modifications of the present invention can facilitate target specificity, the structural tethering of the probe, and its ability to support modular signaling components of greater length or mass. These probe and signaling methods also include means to achieve mixed-color labeling that is specific to each target.

Abbreviations and Definitions

GAP	Gene Amplification Probe
GAP-LOCK	Dual probes locked together by ligation
WRAP-PROBE	Helically wrapped probe that can attach multiple reporters, chained reporters or branched reporters
GENE-TAG	Linear reporter molecules that can attach end to end in chained or branched configurations
TINKER-TAG	GENE-TAG reporters constructed by partially overlapping oligonucleotides that join end to end and that have side arms for joining oligonucleotides bearing label units
COLOR-TAG	GENETAG reporter with a specific labeling or a mix of specific labeling and an associated identifying linker
RING-TAIL	Polynucleotide complex that joins probe components into a potential ring structure and provides end linkers for reporters

RING-LOCK	Complex of GAP-LOCK probe unit and RING-TAIL unit to produce a high specificity ligated circular probe with linkers for reporter attachment
WRAP-LOCK	Complex of WRAP-PROBE unit and RING-TAIL unit to produce a circular probe with linkers to attach reporters
DOUBLE-LOCK	Double RING-LOCK or DOUBLE-LOCK probes confirm mutation detection by targeting sense and antisense strands using different labels on each pair of probes
LINKER	a single stranded nucleotide segment, that is not complementary to the target sequences, and which provides a means to bind probe and reporter components together by virtue of complementary sequences in the linker
Multi-LINKER	a single polynucleotide or a composite or two or more polynucleotides and other matter which has one or more proximal linkers that bind to a probe element and two or more distal linkers that bind to reporter elements such as GENE-TAGs.
COLOR-LINKER	a polynucleotide having a sequence to the central sequence of the GENE-TAG of a particular color
TERMINATOR	a simple oligonucleotide that is complementary to a distal linker and that blocks further chaining of GENE-TAGs, TINKER-TAGS or other linear arrays
TA SITE	nucleotide sequence reading 5' to 3': thymidine, adenine
C9	A spacer, which is nine carbon atoms
PCR	Polymerase chain reaction to amplify DNA
FISH	Fluorescent in situ hybridization
CROSSLINK	covalent linkage between hybridized nucleic acid strands
HYBRIDIZE	formation of specific hydrogen bonding interactions between nucleic acid strands of complementary
PUVA	psovalen plus UVA

Brief Description of the Figures

FIG.1: GAP-LOCK Probe method. 1A depicts dual probes each synthesized with a distal linker. 1B depicts hybridization of dual probes to target sequence in tandem. 1C depicts an alternative construction of the GAP-LOCK probe where one probe is made with a reversing linker in order to provide dual probes having a linker end of the same orientation. 1D depicts a three dimensional view after dual probe ligation showing a helically wrapped configuration. 1E depicts alternate binding of single reporters or a chain of reporters to each GAP-LOCK probe distal linker.

FIG.2: The GAP-LOCK method. 2A depicts an alternative GAP-LOCK probe construction wherein one or more probes are prejoined to PCR generated reporters. 2B depicts hybridization of dual probes to target sequence in tandem 2C depicts a bar graph of the results of Example 1, showing high retention of ligated GAP-LOCK probes with long reporters even after denaturation. 2D depicts an alternative GAP-LOCK probe construction with dual probes on either end of same template. 2E depicts an alternative GAP-LOCK construction wherein one or both probes are prejoined to reporter DNA sequence having a terminal linker for joining additional generic reporter DNAs. FIG. 2F depicts an alternative GAP-LOCK method, the Capture GAP-LOCK method, with a capture moiety joined to the distal end of one probe and not a reporter element, and a reporter element bound to the distal end of the other probe, suitable for use in liquid hybridization conditions and applicable to any formats.

3A: Depicts construction of a typical synthetic WRAP-LOCK probe. 3B depicts an alternative synthetic construction to provide 3' ends and alternative means to use longer probe elements made enzymatically. 3D depicts hybridization of the WRAP-PROBE to target. which provides a helically wrapped configuration.

FIG.4: GENE-TAG method. 4A depicts manufacture of GENE-TAG reporters by PCR. 4B depicts chaining of GENE-TAGs and length control by ratio mix with Terminal-TAGs or Terminator oligonucletides. 4C depicts hybridization of GENE-TAGs to WRAP-PROBE to provide amplified signaling.

FIG. 5: GENE-TAG method. 5A depicts the results of the two experiments of Example 4 with WRAP-PROBEs and different sized chains of GENE-TAGs. 5B depicts construction of synthetic GENE-TAG chains using overlapping oligonucleotides and length controlled by ratio mixing with Terminator oligomers.

FIG. 6: TINKER-TAG method. 6A depicts construction of synthetic GENE-TAGs made with side arms for secondary attachment of label agents. 6B depicts assembly of TINKER-TAG chains that are length controlled by ratio mixing with Terminator oligomers.

FIG. 7: Duo/Layered GENE-TAG method. 7A depicts construction of two types of GENE-TAGs with alternating non-complementary linker pairs so that one type will layer on the other but will not form chains in solution. 7B depicts successive application of the Type I and Type II GENE-TAGs to grow signaling as needed.

FIG. 8 depicts comparison of pre-joined and layered GENE-TAGs applied to WRAP-PROBEs. The WRAP-PROBE for 1-LO-1 gene, provided in different concentrations, binds to target samples on membrane mini-arrays known as Reverse Dot Blots which simulate the conditions of cDNA chip arrays. 8A depicts concentration dependent binding and labeling of probe to 15-LO-1 targets with chained GENE-TAGs made with ratio of 3 GENE-TAGs per terminator. 8B depicts concentration dependent binding and labeling of probe to 15-LO-1 targets with layered GENE-TAGs wherein adding successive GENE-TAG layers of Type I, II and I provides increasing signaling that contrasts with that of chained GENE-TAGs.

FIG. 9: Double-Duo GENE-TAG method. 9A depicts construction of two types of GENE-TAGS with alternating, non-complementary, linker pairs with two distal linkers. 9B depicts successive application of Type I and Type II GENE-TAGS that amplifies two fold with each layer.

FIG. 10: COLOR-TAG method, a variation of the GENE-TAG method allowing multi-colored reporters that bind to specific probes in different proportions giving each probe a distinct signature color. 10A depicts manufacture of different GENE-TAGs to give different colors. 10B depicts construction of three different WRAP-PROBEs for this method and representing three of the four probes needed for Example 12.

FIG.11: COLOR-TAG method. 11A depicts synthesis of COLOR-LINKERS. 11B depicts assembly and joining of first probe (A) with COLOR-LINKER only containing "red" acceptor sequences. 11C depicts similar assembly of second probe (B) with COLOR-

LINKER containing only "green" acceptor sequences. 11D depicts assembly of a third probe (C) with COLOR-LINKER containing mixed "red" and "green" acceptor components.

FIG.12: COLOR-TAG method. 12A depicts hybridizing of the three probes of FIG. 11, containing variant COLOR-LINKERS, to their respective targets. 12B depicts applying a mix of COLOR-TAGs to label each of the three probes differently and simultaneously in order to map the location of each gene or sequence targeted.

FIG. 13: Cells with fluorescent in situ hybridization (FISH) labeling of WRAP-PROBEs for gene sites with "Green" only COLOR-TAGs, "Red" only COLOR-TAGs, and two mixed "Red" and "Green" COLOR-TAGs using method of Example 12. "Red" = rhodamine antibody labeling of digoxygenin haptens, "Green" = fluorescent antibody labeling of biotin haptens.

FIG.14: Multi-LINKER method. 14A depicts the elemental Multi-LINKER structure having a minimum of three linking sites, one proximal and two distal, on one molecule. 14B depicts a composite Multi- LINKER structure to provide a WRAP-PROBE with 8 GENE-TAG binding linker sites on each end of the probe.

FIG.15: Multi-LINKER method. 15A depicts a pair of composite Multi-LINKERs providing multiple binding sites for short oligonucleotides with prejoined labels, such as Cy3 and Cy5, that cannot efficiently label GENE-TAGs or standard probes by enzymatic means. 15B depicts assembled Multi-LINKER and labeled oligomers being added.

FIG.16: GAP-LOCK Capture Probe method. 16A depicts assembly of one probe with distal capture moiety and a second probe with a distal TINKER-TAG reporter. 16B depicts hybridization and ligation of the dual probe components with target strand. 16C depicts capture of ligated GAP-LOCK with TINKER-TAG attached and the application and detection of aequorin conjugated Label subunits to discriminate sequence targeted.

FIG.17: RING-LOCK probe method to encircle the target site and detect single base changes. 17A depicts synthesis and assembly of dual probe and RING-TAIL components. 17B depicts hybridization and ligation of probe to form closed circle; and application and detection of reporters.

FIG. 18: WRAP-LOCK probe method, to avoid ligation step of RING-LOCK method. 18A depicts synthesis and assembly of WRAP-PROBE with RING-TAIL

components. 18B depicts hybridization of probe and application of overlapping Lock component to close loop as well as application and detection of reporters.

FIG. 19: DOUBLE-LOCK probe method, to confirm mutation or sequence detection. 19A depicts preparation of two probes with different linkers that target the sense and antisense strands of the same target site. 19B depicts application of Type 1 reporters to the sense probe and Type 2 reporters to the antisense probe such that low frequency mutations or alterations can be detected.

FIG. 20: GOLD-TAG method depicts gold particles conjugated to short oligonucleotides and joined to TINKER-TAG units, with the labeled TINKER-TAGs then applied to a WRAP-PROBE and target complex. Silver is then precipitated onto gold particles to form large clusters of silver grains, which may then be detected.

FIG. 21A depicts use of the WRAP-PROBES with other commercially signaling units, such as dendrimers and bDNA or multimers. 21B depicts the result of Example 9 showing sensitive detection of GAP-LOCK First Probes with aequorin labeling.

Detailed Description of the Invention

The present invention relates to a number of probe components, multilinking components and signaling components. In a primary embodiment, the present invention relates to a reporter, known as a GENE-TAG, suitable for joining to a probe either alone or in combination with another GENE-TAG reporter, comprising a labeled, double-stranded polynucleotide sequence having one or more first linkers, said first linker comprising a single-stranded nucleotide sequence hybridizable to a complementary single-stranded nucleotide sequence, whereas said first linkers is not hybridizable to a target sequence of said probe. In a further embodiment, the GENE-TAG of the present invention also has one or more second linkers opposed to said first linker, said second linker comprising a single-stranded nucleotide sequence hybridizable to a complementary nucleotide sequence, wherein said second linker is not hybridizable to a target sequence of said probe.

The first and second linkers of the GENE-TAG may or may not be complementary. Where the first and secon linkers are complementary, a reporter array suitable for joining to a probe is formed, comprising two or more of the GENE-TAGs, linked together end-to-end by

hybridization of the second linker of a first GENE-TAG to the first linker of a second GENE-TAG, and optionally, additional GENE-TAGs linked to the remainder of the array by hybridization of the first linker of each subsequent GENE-TAG to the second linker of the preceding GENE-TAG in the array, to form a chained or branched configuration having one or more terminal ends, said terminal end comprising a second linker of a terminal GENE-TAG. The reporter array may also have a terminator at one or more of the terminal ends, said terminator comprises a single-stranded nucleotide sequence complementary to the second linker of one or more terminal GENE-TAGs.

Where the first and second sequence of the GENE-TAG are not complementary, a reporter array is formed using two types of GENE-TAGS. The array is formed by end-to end hybridization of the second linker of a first GENE-TAG to the first linker of a second GENE-TAG, and hybridization of the second linker of a second GENE-TAG to the first linker of said first GENE-TAG, and optionally, additional non-complementary GENE-TAGs linked to the remainder of the array by hybridization of the first linker of each subsequent GENE-TAG to the complementary second linker of the preceding GENE-TAG in the array, to form a chained or branched configuration. When the first and second reporter linkers are not complementary, it is unecessary to use a terminator.

The present invention also relates to a probe component, suitable for hybridizing to a target nucleotide sequence, said probe comprising a first terminal linker, a second terminal linker, and a central sequence complementary to said target nucleotide sequence, wherein said first and second terminal linkers comprise single-stranded nucleotide sequences hybridizable to a complementary nucleotide sequence, but not hybridizable to the target nucleotide sequence of said probe. The present invention also relates to composite probes which can be joined tightly to a particular target strand or sequence, said composite probes comprising at least one probe, said probe comprising a polynucleotide sequence complementary to said target nucleotide sequence, said composite probe further comprising a polynucleotide ring unit, wherein said polynucleotide ring unit is joined to said probe to form an incipient closed loop.

The composite probe of may a dual probe component, wherein a first probe comprises a sequence complementary to a first region of said target nucleotide sequence, and wherein a second probe comprises a sequence complementary to a second region of said target

sequence, wherein said ring unit is joined to said first probe and to said second probe, thereby forming an incipient closed loop.

The present invention also relates to the design and implyment of interconnecting polynucleotide compositions, called MultiLinkers, designed to join, align or multiplex different probe and reporter units together. The Multi-LINKER comprises a first polynucleotide comprising a first terminal linker, a second terminal linker, and at least one internal linker, wherein said first terminal linker, and at least two of said internal and second terminal linkers are hybridizable to complementary nucleotide sequences, and wherein said first and second terminal linkers and said internal linker are not hybridizable to a target sequence of said probe. The Multi-LINKER may also have multiple additional polynucleotides, said additional polynucleotide comprising a first terminal linker, at least one internal linker, and a second terminal linker, wherein the first polynucleotide is joined to the additional polynucleotides by hybridization of the additional polynucleotide to the internal or second terminal linker of the first polynucleotide, and wherein said first and second terminal linkers and said internal linker are not hybridizable to a target sequence of said probe.

The present inventiona also relates to a method of detecting a target sequence, known as a GAP-LOCK method, said method comprising:

a) rendering the target nucleotide sequence substantially single-stranded to give a single-stranded target nucleotide sequence;

b) hybridizing the target sequence with a first probe and with a second probe, wherein said first and second probes hybridize at tandem locations on the target nucleotide sequence, wherein each of said first and second probes comprises a proximal ligation end and wherein one or both of said first or second probes further comprises a distal linker, said distal linker comprising a single-stranded nucleotide sequence that does not hybridize to the target nucleotide sequence; wherein said distal linker is joined to one or more reporters, either prior to or subsequent to the hybridization of the first and second probes to the target sequence;

c) ligating the hybridized first and second probes at their respective proximal ligation ends to form a composite probe structure known as a GAP-LOCK probe:

d) joining said reporter or reporters to said distal linker, if not previously joined;

e) denaturing to remove any unligated first and second probe;

f) determining whether the target nucleotide sequence is present by detecting the presence or absence of said reporter or reporters.

In a particular embodiment of the GA-LOCK method,, both the first and second probes have a distal linker. This GAP-PROBE method has been shown to provide stable support for long or multiple reporters.

The GAP-LOCK method of the present invention can also be used under liquid or near-liquid hybridization conditions, wherein said first probe comprises a distal end that is joined to a capture moiety, and said second probe has a second distal linker, wherein the hybridization complex is denatured to remove the target sequence, and capturing the GAP-LOCK probe to detect the reporter. Alternatively, the capture moiety can be joined to the distal linker of a first probe. This embodiment of the GAP-LOCK method is known as the Capture GAP-LOCK method.

The methods of the present invention also contemplate other methods for using of GENE-TAG probes, including composite probes, which hybridize to the probe to form a ring structure or an incipient ring strucutre. In one embodiment, the present invention relates to a RING-LOCK method for providing a circular enclosure of a target strand by a composite probe , said method comprising:

a) providing a target strand comprising a target sequence;

b) providing a GAP-LOCK probe, wherein said GAP-LOCK probe has a first and second distal linker;

c) providing a RING-TAIL Unit comprising at least three single-stranded polynucleotides, wherein at least one single-stranded polynucleotide is joined or suitable for joining to the first distal linker, and wherein at least one single-stranded polynucleotide is joined or suitable for joining to said second distal linker; and wherein at least one single-stranded polynucleotide is joined or suitable for joining to one or more reporters;

d) rendering or treating said probes and said RING-TAIL Unit to effect cross linking or to increase binding;

e) hybridizing the first and second probe to the target, thereby forming a hybridized complex;

f) ligating said first and second probes, thereby forming a closed loop or an incipient closed loop around the target strand;

g) hybridizing at least one single-stranded polynucleotide to said first distal probe linker, if not previously joined; hybridizing at least one single-stranded polynucleotide to said second distal probe linker, if not previously joined;

h) hybridizing a reporter or reporters to at least one single-stranded polynucleotide, if not previously attached, or if necessary;

i) denaturing or washing the hybridized complex to remove unligated probe and reporters;

j) detecting the presence of reporters to indicate the target sequence.

The present invention also relates to a method of simultaneously detecting a target sequence on both a sense and an antisense strand of DNA, known as a DOUBLE RING-LOCK method, comprising:

a) providing a double-stranded DNA comprising a sense target strand and an antisense target strand, wherein said sense target strand comprises a sense target sequence, and wherein said antisense target strand comprises an antisense target sequence;

b) providing a sense GAP-LOCK probe, said GAP-LOCK probe having a first and second distal linker;

c) providing an antisense GAP-LOCK probe, said GAP-LOCK probe having a first and second distal linker;

d) providing a first RING-TAIL Unit comprising at least three first single-stranded nucleotides, wherein at least one said first single-stranded nucleotide is joined or suitable for joining to said first distal linker of said sense first probe, and wherein at least one said first single-stranded nucleotide is joined or suitable for joining to said first distal linker of said second sense probe, and

wherein at least one first single stranded nucleotide is joined or suitable for joining to one or more first reporters;

e) providing a second RING-TAIL Unit, comprising at least three second single-stranded polynucleotides, wherein at least one said second single-stranded polynucleotide is joined or suitable for joining to said first distal linkerof said antisense first probe, and wherein at least one said second single-stranded polynucleotide is joined or suitable for joining to said first distal probe linker of said second antisense probe, and wherein at least one second single stranded polynucleotide is joined or suitable for joining to one or more second reporters, wherein said second reporter produces a signal distinct produces a signal distinct from a signal produced by said first reporter;

(f) treating the probes and RING-LOCK Units, at this step or another step, to effect cross linking or to increase binding;

(g) hybridizing said first and second sense probes to the sense strand, thereby forming a sense hybridized complex; and hybridizing said first and second antisense probes to the antisense strand, thereby forming an antisense hybridized complex;

(h) ligating said first and second sense probes, thereby forming a closed loop or an incipient closed loop around the sense target strand; and ligating said first and second antisense probes, thereby forming a closed loop or an incipient closed loop around the antisense target strand;

(i) joining at least one first single-stranded polynucleotide to said first distal linker of said first sense probe, if not previously joined; joining at least one first single-stranded polynucleotide to said second distal linker of said second sense probe, if not previously joined; joining at least one second single stranded polynucleotide to said first distal linker of said first antisense probe, if not previously joined; joining at least one second polynucleotide to said second distal linker of said second antisense probe, if not previously joined;

(j) joining one or more first reporters to at least one said first single-stranded polynucleotide, if not so previously joined; joining one or more second

reporters to at least one said second single-stranded polynucleotide, wherein said second reporter produces a signal distinct from said first reporter;

(k) determining whether the sense target sequence is present by detecting the presence or absence of said first reporter or reporters; or determining whether the antisense target sequence is present by detecting the presence or absence of said second reporter or reporters; or both.

In an alternative embodiment, the GAP-LOCK method of the present invention involves formation of a ligated dual probe having one or more reporters attached directly thereto. This embodiment of the GAP-LOCK method for detecting a target nucleotide sequence comprises:

a) rendering the nucleotide sequence substantially single-stranded to give a single-stranded nucleotide sequence;

b) hybridizing the target sequence with a first probe and a second, wherein said first and second probes hybridize at tandem locations on the target, wherein each of said first and second probes comprises a proximal ligation end, and a distal end pre-joined to one or more reporters;

c) ligating the hybridized first and second probes at their respective proximal ligation ends to form a GAP-LOCK probe;

d) denaturing or washing to remove any unligated first and second probe;

e) determining whether the target nucleotide sequence is present by detecting the presence or absence of said reporter or reporters.

According to this embodiment of the GAP-LOCK method, the distal end is pre-joined directly to a first reporter comprising a labeled, double-stranded polynucleotide generated by PCR. The first reporter may also have one or more distal reporter linkers, said distal reporter linker comprising a single-stranded nucleotide sequence hybridizable to a complementary nucleotide sequence, said distal reporter linker not hybridizable to the target nucleotide sequence. The distal reporter linker may be suitable for hybridizing to a labeled, double-stranded DNA sequence, known as a GENE-TAG, comprising one or more first reporter linkers, wherein said first reporter linker comprises a single-stranded nucleic acid sequence hybridized to the distal reporter linker of said first reporter.

Multiple reporters may be provided in this embodiment to form of a reporter array, said reporter array comprising a first labeled, double-stranded polynucleotide sequence, known as a GENE-TAG, linked together end-to-end by hybridization to one or more GENE-TAGS, wherein said first GENE-TAG comprises one or more first reporter linkers, said reporter linker comprising a single-stranded nucleotide sequence hybridizable to the distal reporter linker of said first reporter, and one or more second reporter linkers hybridized to one or more first reporter linkers of a second GENE-TAG, and wherein said second GENE-TAG further comprises one or more second reporter linkers, and optionally, additional GENE-TAGs linked to the remainder of the array by hybridization of the first reporter linker of each subsequent GENE-TAG to the second reporter linker of the preceding GENE-TAG in the array, to form a chained or branch configuration having one or more terminal ends, said terminal end comprising the second reporter linker of a terminal GENE-TAG.

The present invention also relates to a method of detecting a target nucleotide sequence, known as the WRAP-LOCK method, comprising:

a) rendering the target nucleotide sequence substantially single-stranded to give a single-stranded target nucleotide sequence;

b) hybridizing the single-stranded target nucleotide sequence with a nucleic acid probe, thereby forming a hybridized WRAP-PROBE complex of a single-stranded target nucleotide sequence and a nucleic acid probe, said WRAP-PROBE comprising a central sequence complementary to the single-stranded target nucleotide sequences, and further comprising a probe linker at one or both terminal ends, said probe linker comprising a single-stranded nucleotide sequence that does not hybridize to the target sequence; wherein said probe linker sequence is joined to one or more reporters, either prior to or subsequent to the hybridization of the probe to the target sequence;

c) washing to remove any unbound probe;

d) joining said reporter to said probe linker, if not previously joined;

e) detecting the presence or said reporter or reporters to indicate the target sequence.

The reporter or reporters linked to the WRAP-LOCK probe may be linked directly or indirectly. In one embodiment of the method of the present invention, a multilinking unit indirectly joins the WRAP-LOCK to one or more reporters.

In a still further embodiment, the WRAP-LOCK method provides for circular enclosure of the target polynucleotide strand with a WRAP-PROBE, which method comprises:

a) providing the WRAP-PROBE having a first and second terminal probe linker;

b) providing a RING-LOCK Unit comprising at least two single-stranded polynucleotides, wherein at least one single-stranded polynucleotide is joined or suitable for joining to said first terminal probe linker, and wherein at least one single-stranded polynucleotide is joined or suitable for joining to one or more reporters;

c) treating the probe and RING-TAIL unit, at this step or another step, to effect cross linking or to increase binding;

c) hybridizing the WRAP-PROBE to the target strand, thereby forming a hybridized complex;

d) joining at least one single-stranded polynucleotide to said first terminal probe linker, if not previously joined,

e) providing a looping nucleotide comprising a first region complementary to said second terminal probe linker, and a second region complementary to said RING-TAIL Unit, wherein said looping nucleotide hybridizes to the second terminal probe linker and the RING-TAIL Unit, thereby forming a closed loop about the target strand;

f) joining a reporter or reporters to at least one ring-tail linker, if not previously attached, or if more or needed; and

g) detecting the presence of reporter to indicate the target sequence.

In a further embodiment, the present invention relates to a method of simultaneously detecting a target sequence on both a sense and anti-sense strand of DNA, known as the DOUBLE-WRAP LOCK method, said method comprising:

a) providing a double-stranded DNA comprising an antisense strand and a sense strand, wherein both said antisense strand and said sense strand comprise a target sequence;

b) providing a sense WRAP-PROBE having a first and second terminal probe linker, said sense WRAP-PROBE probe having a central sequence complementary to the sense strand target sequence;

c) providing an antisense WRAP-PROBE having a first and second terminal probe linker, said antisense WRAP-LOCK probe having a central sequence complementary to the antisense strand target sequence;

d) providing a first RING-TAIL Unit comprising at least two first single-stranded polynucleotides, wherein at least one first single-stranded polynucleotide is joined or suitable for joining to said first terminal probe linker of said sense WRAP-PROBE, and wherein at least one first single-stranded polynucleotide is joined or suitable for joining to one or more first reporters;

e) providing a second RING-LOCK Unit comprising at least two second single-stranded polynucleotides, wherein at least one second single-stranded polynucleotide is joined or suitable for joining to said first terminal probe linker of said antisense WRAP-PROBE, and wherein at least one second single-stranded polynucleotide is joined or suitable for joining to one or more second reporters, wherein said second reporter produces a signal distinct from said first reporter;

(f) treating the WRAP-PROBEs and the RING-LOCK Units, at this step of another step, to effect cross linking or to increase binding;

(g) hybridizing said sense WRAP-PROBE to the sense strand, thereby forming a sense hybridized complex; and hybridizing said antisense WRAP-PROBE to the antisense strand, thereby forming an antisense hybridized complex;

(h) joining at least one first single-stranded polynucleotide to said first terminal probe linker of said sense WRAP-PROBE, if not so previously joined; and joining at least one second single-stranded polynucleotide to said first terminal probe linker of said antisense WRAP-PROBE, if not so previously joined;

(i) providing a first looping nucleotide comprising a first region complementary to said second terminal probe linker of said sense WRAP-PROBE, and a second region complementary to said first RING-TAIL Unit, wherein said looping nucleotide hybridizes and thereby forms a closed loop about the target sense strand; and providing a second looping nucleotide comprising a first region complementary to said second terminal probe linker of said antisense WRAP-PROBE, and a second region complementary to said second RING-TAIL Unit, wherein said looping nucleotide hybridizes and thereby forms a closed loop about the target antisense strand;

(j) joining one or more first reporters to at least one said first single-stranded polynucleotide, if not so previously joined; joining one or more second reporters to at least one said second single-stranded polynucleotide, if not previously joined, wherein said second reporter produces a signal distinct from said first reporter;

(k) determining whether the sense target sequence is present by detecting the presence or absence of said first reporter or reporters; or determining whether the antisense target sequence is present by detecting the presence or absence of said second reporter or reporters, or both.

The method of the present invention also relates to a method for exponential signal amplification, known as the GENE-TAG method, comprising:

a) providing a single-stranded target nucleic acid sequence;

b) providing a probe comprising a sequence hybridizable to said target nucleic acid sequence, and a terminal linker sequence on one or both ends of the probe, wherein said linker sequence is a single-stranded polynucleotide hybridizable to a complementary sequence;

c) providing one or more GENE-TAGs, said GENE-TAG comprising a labeled, double-stranded polynucleotide reporter having one or more first reporter linkers and one or more second reporter linkers opposed to said first reporter linker, said reporter linkers comprising single-stranded polynucleotide sequences not hybridizable to the target sequence, wherein at least one said

GENE-TAG has a first reporter linker complementary to said probe terminal linker, and wherein one or more said reporter linkers link together end-to-end by hybridization of the first reporter linker of each subsequent GENE-TAG to the second reporter linker of each preceeding GENE-TAG in the array, to form a chained or branched configuration comprising one or more terminal end, wherein said terminal end comprises the second reporter linker of a terminal GENE-TAG; wherein said GENE-TAG is joined to said probe and this or a subsequent step;

d) hybridizing said probe to said target nucleotide sequence;

e) joining said GENE-TAG or GENE-TAGs to said probe, if not so previously joined;

f) determining the presence of the target sequence by detecting the presence of the GENE-TAG or GENE-TAGs.

In one embodiment of the GENE-TAG method, the first and second reporter linkers of the GENE-TAGs are complementary, such that only GENE-TAGs of one type are required to form chained or branched reporter arrays. The length of said chained or branched reporter arrays, and thus probe amplification, may be controlled controlled by the ratio of GENE-TAGs to said terminators. A Terminator TAG represents one such terminator, said Terminator TAG comprising a GENE-TAG having no second reporter linker, wherein said first reporter linker of said Terminator TAG hybridizes to the second reporter linker of a terminal GENE-TAG of the chain or branch.

In an alternative embodiment of the GENE-TAG method, the first and second reporter linkers of the GENE-TAG are not complementary. According to this embodiment, the method requires at least two tyoes of GENE-TAGs to form chained or branched arrays, wherein GENE-TAGs of a first type comprise a first reporter linker complementary to the probe terminal linker, and a second reporter linker complementary to the first reporter linker of GENE-TAG of the second type, and wherein said GENE-TAG of the second type has a first reporter linker complementary to the second reporter linker of a GENE-TAG of the first type, such that only a GENE-TAG of the first type will bind to a GENE-TAG of the second type, and only a GENE-TAG of the second type will bind to a GENE-TAG of the first type; wherein said chained or branched arrays are formed by providing GENE-TAGs sequentially,

alternating between the first and second type, and thereby forming alternating layers of GENE-TAGs of a first or second type upon the probe.

GENE-TAGS are constructed by PCR amplification of arbitrary template DNA using one or more modified oligonucleoties as primers, wherein each modified oligonucleotide has one or more internal spacers, whereby the first and second terminal single-stranded linkers are preserved. Alternatively, GENE-TAGs are constructed by hybridization of two polynucleotides, said first polynucleotide having a second terminal end complementary to the first terminal end of a second polynucleotide. TINKER TAGs represent the GENE-TAG structure formed when the first polynucleotide has an internal sequence complementary to the internal sequence of a second polynucleotide, forming a GENE-TAG having side arms, said side arms comprising the second terminal end of the first polynucleotide, and the first terminal end of the second polynucleotide. These TINKER-TAGs may be labled indirectly, by binding short oligonucleotides bearing label units.

The present invention also relates method of constructing probes detectable on the basis of a unique signal, which method permits simultaneous detection of more than one probe. This method, known as the COLOR-TAG method, comprises:

a) providing a probe hybridizable to a single-stranded nucleotide sequence, wherein said probe comprises a terminal linker at one or both ends, said terminal linker comprising a single-stranded polynucleotide capable of binding to a complementary sequence;

b) preparing on or more COLOR-TAGs, said COLOR-TAG comprising a double-stranded polynucleotide reporter having a first reporter linker and a second reporter linker opposed to said first reporter linker, wherein said COLOR-TAG is labeled with one or more labels or colors, or both, and wherein said first and second reporter linkers are specific to the labels or colors employed; wherein at least one COLOR-TAG terminal linker is hybridizable to the terminal linker of said probe;

c) hybridizing one or more said COLOR-TAGs to the probe, at this step or a later step;

e) hybridizing said probe to said target sequence;

f) hybridizing said COLOR-TAG to the probe, if not so previously

hybridized;

f) determining the presence of said target sequence by detecting the presence of said COLOR-TAG.

In one embodiment of the present invention, the COLOR-TAG method may also include an intermediate multilinking structure, linking the probe to two or more reporters. This multilinking structures serves to increase the number of COLOR-TAGs that can be joined to a given probe, and therefore the possible COLOR-TAG combinations that can be used to unqiuely identify a probe. The multilinker structure contains one or more color acceptor sequences, called COLOR-LINKERS, which sequences are specific to the linkers of a particular COLOR-TAG. In this way, multilinkers can be designed to attract particular COLOR-TAGS, to give a characteristic label to a particular probe. The COLOR-TAG may have two or more different labels or colors associated therewith.

The present invention relates to a new class of reagents and methods for detecting nucleic acid sequences comprising complexes of synthetic probes and generic reporter molecules in which the nucleic acid probe component recognizes and binds to the target sequence by hybridization, but it is helically wrapped around the target, and it is bound to one or more polynucleotide reporter molecules from one or both ends, wherein the probe and reporter complex are thereby tethered to the target strand by forces which exceed that of simple hybridization. This configuration, and alternate configurations which more completely encircle the target, and/or which provide an multiple linking component, provide a basis for the attachment of extended and multiple polynucleotide reporters, in chained or branched configuration, which can be constructed beforehand, which are composed of synthetic oligonucleotides and or modified amplicons of deoxyribonucleic acid which have been made or rendered partially single stranded on their termini so that they can link up end to end. Various components of these probe and reporter systems as well as probe and target complexes are more firmly joined together by covalent crosslinking or by employing artificial nucleotide bases with greater binding capacity. In addition, the hybridized linkages that will occur between probe and reporter components, or between an interposed multilinking structure and these components, are designed such that these bonds will generally pull end to end rather than laterally under the conditions employed. This structural principal sums the hydrogen bonds between linkers together in the same manner that the hooks and eyes of

joined Velcro strips have amplified binding when pulled end to end versus laterally. These novel reagents and methods provide multiple modular systems for detecting and mapping genes and genetic components, for quantitating gene expression, for detecting mutations and chromosomal alterations in cancer and genetic diseases, and for discriminating and diagnosing bacterial and viral infectious agents.

The principal probe methods and compositions are based on the GAP-LOCK and WRAP-PROBE designs, including the Capture-GAP-LOCK method, and the principal signal amplification methods and compositions are based on the GENE-TAG and COLOR-TAG designs.

GAP-LOCK Technology

The GAP-LOCK design entails two probes, one with a 5' head end and the other with a 3' head end, that hybridize to adjacent tandem sites on the target and that carry extended or multiple reporter structures or have the capacity to attach such reporters. If the match is exact between the probe and target, in the bases immediately near the gap between the probes, then they can be ligated together. Successful ligation confers a centralized, structurally-wrapped configuration that effectively tethers the probe and the extended reporters to the target site and stabilizes them against washing and/or denaturation. Signal retention thus indicates detection of the mutation or base change targeted, while failure to ligate, prevents or leads to loss of signal and indicates the absence of the target sequence. Consequently, when this invention is applied, it provides the simultaneous advantage of improving both the detection of small base changes and the amplification of signaling.

GAP-LOCK probes are manufactured in several modifications, the primary embodiment being two probes with distal linkers, with reporters linked to the probes afterwards or beforehand. In one embodiment, one or both of the probes are prejoined to PCR-generated reporters. See Figure 2A. This method demonstrated preferential retention of ligated probes with long reporter tails after alkali treatment. See Figure 2C. In a particular embodiment, the reporter may have one or more terminal linkers for binding one or more reporters. See Figure 2E. In further embodiment of the invention, one or both probes are synthesized having a distal terminal linker for the attachment of a reporter having a complementary sequence, prior to or subsequent to probe hybridization. See Figure 1A. Alternatively, effective GAP-LOCK probes have been manufactured wherein the dual probes

have been joined to the same double-stranded reporter, with the 5' probe on one end and the 3' probe on the other end. See Figure 2D. With formats that approximate or favor liquid hybridization conditions, such as microwell plates, and with small or unstable targets, such as DNA or RNA samples used with various DNA chip formats (oligonucleotide arrays, optical chips, cDNA arrays), these probe subunits can be constructed so that one probe linker can be affixed with capture moieties, to complex the probe to a substrate, while the other probe linker can be affixed with reporters. See Figure 2F. With this modification, the target strand can be removed after hybridization and ligation, leaving a ligated GAP-LOCK complex intact to capture the reporter and to provide definitive signaling.

WRAP-PROBE Technology

The WRAP-PROBE design extracts the central structural feature of the GAP-LOCK design, namely, the centralized, helically-wrapped probe configuration of the method, which effectively wraps the ligated probe complex around the target site and joins one or more reporters from one or both sites. However, this embodiment involves a single probe, avoiding the need for ligation. Therefore, the probe complex consists of a target specific segment of nucleic acid sequences, midway within the probe, and attachment sites or linkers at one or both ends of the probe. After the probe component is hybridized to the target site, one or more reporters are linked directly to the ends of the probe or to intermediate multilinking structures. This helical and linear orientation of the probe, of the linkers, or multilinker structures, and of the reporters are designed to both stabilize the probe and to support multiple or highly extended reporters. At the same time, the need for ligation is eliminated. This design can therefore improve specificity, it allows exponential signaling, and it simplifies the manufacture of new probes for different targets.

The WRAP-PROBE units were synthesized as two overlapping oligonucleotide subunits which are joined together. See Figure 3B. The oligomers (and all subsequent oligomers for probe and reporter components) were made with a DNA synthesizer using phosphoramidite chemistry. The primary probe subunit contains first linker sequences on the 5' end which are complementary to the reporter, target specific sequences in the mid-portion, and second linker sequences on the 3' end which are complementary to the secondary probe subunit. To facilitate hybridization, small sets of spacer bases (ie: TTT) are commonly included in the primary probe subunit to slightly separate the target specific segment from the

two linker segments. The secondary probe subunit is generic and lacks target specific sequences, while its 3' end is complementary to the second linker sequences of the primary probe subunit, and its 5' end contains first linker sequences complementary to the reporter unit. The 3' linker sequences are designed with 5' TA sites to facilitate psoralen plus UV crosslinking. Such crosslinking employs a bi- or tri-functional reactive psoralen compound which intercalates between paired bases, and, upon exposure to long wave UV light (blacklite), forms a covalent bridge between thymidine bases that are adjacent to but opposite one another on facing complementary strands.

Alternatively, the probe unit can be made with 3' linker ends or as a single oligomer which is synthesized with linker sequences on both ends and target specific sequences in the middle. In the latter case, each end will attach reporters differently. Such WRAP-PROBE units can also be manufactured by cloning or PCR amplification of a target specific segment to which linkers on one or both ends are attached by a second annealing/hybridization step similar to the attachment of the linker subunit in the principal WRAP-PROBE method described above. See Figure 3C.

The present embodiment of a WRAP-PROBE entailed a structure which is complementary to the target sequences in the central region of the probe unit and each terminal region contain a probe linker sequence for reporter attachment, or attachment of a multilinking unit. See Figure 3B. This probe is distinguished by the initial formation of a probe that wraps around the targeted sequences at midpoint (Figure 3D) and by the secondary binding of reporters to the terminal linkers such that the reporters are thereby structurally tethered to the targeted location.

GENE-TAG Technology

The GENE-TAG design extracts the reporter component of the GAP-LOCK design and makes it a generic product capable of attaching to any WRAP-PROBE, to any GAP-LOCK probe modified to accept such reporters, or to any other probe design that provides appropriate generic linkers for attachment. The typical embodiment of the GENE-TAG reporters are extended segments of arbitrary reporter DNA with terminal linker ends that can attach to the probe and/or to other such reporters. See Figure 4A. Depending on the sequences or the nature of the linkers employed, such reporters can form extended chains or branching structures of such reporters. See Figure 4B. These reporter arrays can then bind to

a hybridized probe such as a WRAP-PROBE and produce signaling which is largely proportional to the length and density of the reporter arrays. See Figure 4C and 5A. The invention also includes several modifications of this design to suit different requirements and conditions as well as procedures to limit or control the growth of reporter arrays. These GENE-TAG components can also be manufactured by different processes. The primary embodiment is based on PCR amplification using spacer modified oligonucleotides as primers to produce single stranded linkers on either end of a generic reporter template. See Figure 4A. However, means are also devised to create similar structured reporters assembled from overlapping oligonucleotides. See Figure 5B. One version of this design, also known as the TINKER-TAG method, provides secondary linker side arms to allow interchangeable labeling elements that are applied separately. See Figure 6A and 6B. The key aspect of all these methods is the facility for modular, amplified signaling.

The PCR generated reporters of the present invention generally consists of long segments of labeled double-stranded DNA (generally 100 - 800 bp) with short single-stranded tails (generally 20 - 30 bp) on one or both ends to serve as linkers. The base pair sequences within the long double-stranded portion are arbitrary and these bases only serve to carry signaling elements. In contrast, the single-stranded tails have specific linker sequences that are complementary to the probe unit, to other reporter units, or to a multilinking structure. These GENE-TAG reporters were manufactured with staggered single-stranded linkers at each end wherein the first or proximal linker is complementary to the linker sequence of the probe, and wherein the second or distal linker is complementary to the first linker of the same GENE-TAG or another GENE-TAG. While this design would theoretically allow the ends of a reporter or reporter chain to self join as a closed circle, such events are largely prevented by the limited flexibility of double-stranded DNA. Thereafter, these complementary double-tailed reporters generally join to one another on an end-to-end basis, forming a linear chain that amplifies signaling. A smaller proportion of said reporters were constructed with only one single-stranded end, hereinafter termed Terminal TAGs, and this end contains sequences that are complementary to both the probe unit and to the second linker of a GENE-TAG. These one-ended Terminal TAG units serve to cap a growing reporter chain or to simply provide a singular reporter unit. Chains of GENE-TAG units were made to statistically predictable length by mixing a proportion of Terminal TAGs in with an equal or larger

concentration of double-ended GENE-TAGs. Figure 4B. While some variability in chain length will occur with this method, the relative length of the reporter chain can be determined by the ratio of GENE-TAGs to Terminal TAGs employed. Alternatively, reporter chains can be capped and controlled in length by ratio mixing GENE-TAGs with a simple oligonucleotide, called a TERMINATOR, having the same sequences as the linker of a Terminal TAG.

Alternatively, GENE-TAGs may be designed to form a defined reporter chain using a series of paired linker sequences between TAG units such that the first reporter would join to the probe, the second reporter would sequentially join to the first reporter, the third reporter to the second reporter, and so on. See Figure 7. These reporter chains could then self-assemble in place, they could be prejoined, or they could be added sequentially as needed to provide the required signaling. With this method, Terminal TAGs or Terminators would be unnecessary. Such defined chaining was accomplished using only two types of GENE-TAGS (Type I and II) that were sequentially added in layers. In this embodiment, the proximal and distal linkers of each type of GENE-TAG are not complementary to one another. Rather, the TYPE I GENE-TAG has a proximal linker that attaches to the probe, and a distal linker that attached to the proximal end of a Type II GENE-TAG. Whereas a Type II GENE-TAG has a distal linker that will bind to the proximal end of a Type I GENE-TAG. Thus, a Type I will only bind to a Type II and a Type II will only bind to a Type I, so that when kept separate they will not bind to form chains. Thus, a chain is created by layering, with the probe applied first, Type I GENE-TAGs next, Type II next, and back to Type I, with a simple wash between successive GENE-TAG applications. See Figure 7B. It was found that three such layers gave labeling superior to a premade group of standard GENE-TAGS made by ratio mixing three reporters to one terminator. See Figure 8. Alternatively, the distal ends of the Type I and Type II GENE-TAGs can be made with two or more distal linkers, such that each layer will exponentially bind more GENE-TAGs. See Figure 9.

GENE-TAGs and Terminal TAG reporter units were made using modified oligomers as primers to amplify arbitrary template DNAs. The template DNAs are typically derived from cloned inserts of M_{13} or other plasmids such as pGEM3, and the size of the inserts (typically 100 to 800 bp) determines the length of the resulting reporter unit. The modified oligomers were synthesized as linear products with three functional regions: a 5' end

containing 20-30 nucleotides of a linker sequence, an mid-section of two C9 spacers, and a 3' end containing 15-25 nucleotides of a M13 forward or reverse primer sequence. These C9 spacer phosphoramidites (Glen Research) insert a mixed polarity 9 atom carbon spacer arm within the oligonucleotide chain. The modified oligonucleotides were purified by centrifuge filtration with OPC columns (ABI). Linker sequences were screened to ensure effective binding and to avoid repetitive sequences or unique sequences that may occur in targeted genomes. These linker sequences may also be designed with one or more 5' TA sites to facilitate psoralen crosslinking or with other sequences suitable for other crosslinking agents. In other experiments, linkers with a 5' terminal end were designed with an initial 5' TA sequence and synthesized with a C2 psoralen phosphoramidite at the 5' end. This modification ensures efficient crosslinking between linkers since it tethers a psoralen molecule to be exactly positioned at a potential crosslinking site.

For GENE-TAG manufacture, two modified oligomers were synthesized using one linker sequence and M13 forward sequences on one oligomer, and using M13 reverse sequences and a different or complementary linker sequence on the other oligomer. For the Terminal TAGs only one modified oligomer was made with linker sequences complementary to the probe on the 5' end and with M13 forward sequences on the 3' primer end. While the 3' end of one of these modified oligomers acts as a primer during standard PCR reactions, the single-stranded linker segment that is located 5' of the C9 spacers remains single-stranded during PCR amplification since the polymerase cannot jump across the carbon spacer region. In making Terminal TAG reporters by PCR, the modified oligomer only substitutes for one primer, such as M13 forward, in which case, a standard M13 reverse oligomer is used for the second primer. In making GENE-TAGs, the two modified oligomers substitute for both the forward and reverse primers. Figure 4A. In addition to M13 based inserts, a variety of other DNA templates and similarly modified primer pairs are suitable for making similar labeled reporter units of variable length.

COLOR-TAG Technology

The COLOR-TAG design is based on the GENE-TAG method and reporter compositions, however it uses GENE-TAG reporters with different color or signaling labels to achieve probes with specific color signatures. See Figures 10,11,12 and 13. In one

embodiment, this method created reporters with a solid color or with a mix of colors, that was sufficient to identify specific probes, by employing two or three generic GENE-TAG reporters of different color called COLOR-TAGs. These reporters are joined to each probe by virtue of an intermediate polynucleotide structure of multiple linkers, called a Multi-LINKER, that defined the mix and proportion of each COLOR-TAG that will bind. More particularly, the Multi-LINKER contains one or more linkers, known as COLOR LINKERS, having a particular color acceptor sequence, which acceptor sequence is complementary to a COLOR-TAG bearing a particular colored label. Thus, color variations are achieved by changing the mix of linkers or the proportion of one linker type over another which comprise the Multi-LINKER. When WRAP-PROBEs were employed, Multi-LINKER structures were affixed beforehand to the probe or added as an intermediate step before adding pre-made chains of reporters. After hybridizing such probes and linkers to their target sequence, the resulting complex becomes a molecular trap for a predicted mix of COLOR-TAGS that will define the underlying probe. In this manner, a variety of different probes were employed and differentiated at the same time. See Figure 12 and 13. Alternatively, similar color or labeling differentiation could be achieved by preparing COLOR-TAGs with a distinct mix of colored labeling within them, and with a linker sequence at the proximal end that specifies which color mix will occur when attached to the linker of a particular probe. Thus, different COLOR-TAG "flavors" can be created and employed.

Multi-LINKER Technology

Polynucleotide structures have been devised that provide one or more proximal linkers that bind to a probe component and two or more distal linkers that bind to a reporter element, such as GENE-TAGS. These interposed structures, known as Multi-LINKERS, greatly increase the signaling potential of a particular probe. Multi-LINKERS can take the form of single polynucleotides or a composite of two or more polynucleotides.

The single polynucleotide form represents the most elemental Multi-LINKER, as depicted in Figure 14A. wherein a oligonucleotide has one proximal linker and two identical distal linkers on the same molecule. This elemental Mutli-LINKER has the minimal capacity to join two reporters to one probe. This elemental structure can also take a form wherein carbon spacers are introduced during synthesis, as depicted to the right of Figure 14A, so that

greater flexibility is achieved and the binding sites are more accessible. These elemental Multi-LINKERs are based on the ready ability of commercial synthesizers to make long oligonucleotides. Consequently, fundamental units can also be designed and created with sufficient length to provide a proximal linker site on one termini and a larger series of distal linker sites arranged in tandem on the other end of a single polynucleotide. Such distal linkers could also have different rather than identical sequences.

Composite polynucleotide Multi-LINKER structures have also been devised, comprising two or more polynucleotides. These composite polynucleotides are made of partially overlapping oligonucleotides, that are hybridized and crosslinked together, to create a complex of linkers joining one probe to two or more reporters. See FIG. 14B. These composite structures may be in branched or chain form. The chained multi-linker structure of the COLOR-TAG method is one such embodiment and this employs a few generic oligonucleotide components that partially overlap and that repeatedly link together in a linear array.

Such chained multi-linker structures do not have an exact length and the number of repeated linker elements in the array is dependent upon the proportion of terminators provided when mixing the linker components together in hybridizing conditions. Since the intended chain length is created by ratio mixing, individuals chains formed in solution are expected to vary considerably, and the mix ratio employed, such as 4:1, simply provides an estimate of average length. Alternatively, the elemental Multi-LINKER structure shown in FIG. 14A provide a defined set of linker elements and thus bind a more predictable number of reporters.

Multi-LINKERs are designed on the same principles employed in the present invention for joining probes and reporters together by their complementary linking sequences, generally called linkers. Namely, the sequences and polarity of the linkers are arranged so that they will generally pull on an end-to-end basis under typical hybridizing and wash conditions. Thus, if the probe linkers have 5' termini, the complementary Multi-LINKER proximal linker will also be 5', and the same alignment matching is employed with 3' linkers, or when the probe and reporter on the other end of the interposed Multi-LINKER differ in alignment. Furthermore, the lengths and compositions of the linker segments are constrained by any need to join elements together in situ versus situations where they can be prejoined or

crosslinked. When prejoining and crosslinking can occur, short segments of 10 to 20 bases are commonly used and these are generally designed with 3 to 5 dispersed sites containing 5' TA sequences to allow the incorporation of free psoralen and crosslinking with near UV (PUVA). However, longer linker segments of 20 to 30 bases are generally employed where linker recognition must occur with sample DNA or RNA present and where crosslinking may not be employed or should be avoided. Nonetheless, to allow alternate modular use of the same components for other probe and or signaling constructions, the longer linkers are still designed with approximately five 5' TA sites and both short and long linker sequences are designed with very similar GC content generally in the range of 45 to 65%. Linker sequences are also checked against known sequences expected in the target sample. Furthermore, common limits of oligonucleotide synthesis indicated that capped shortmers occur at approximately a one percent rate per each base of length, and thus, Multi-LINKER components are generally designed to maximize short components up to 50 bases and to limit long components to about 80 bases.

Based on the above principles, the composite Multi-LINKER depicted in FIG. 14B and 14C is designed as the simplest complex that can readily be achieved to reliably join a large number of GENE-TAGs, having 5' linkers, to probes with 5' ends such as the WRAP-PROBES of the present invention. The first unit (ONE-TO-FOUR) provides four fold amplification of short distal linkers and the second unit (ONE-TO-TWO) adds two fold amplification by providing 2 longer linkers per unit. Thus, each end of the WRAP-PROBE will join about 8 GENE-TAGS to it via the Multi-LINKER with all components being aligned in a linear manner. Additionally, layered GENE-TAGS with double distal linkers, as shone in FIG.9, could also be used so that the second layer would bind another 16 GENE-TAGs to each probe end. Since two such layers could be readily applied, this embodiment would provide approximately 50 GENE-TAGS per probe.

In addition to providing distal linkers that bind GENE-TAGS, such Multi-LINKERs can also provide multiple binding sites for other types of reporters, including short oligonucleotides that are prejoined to labeling agents either during synthesis, or by secondary chemistries, such as amine or thiol linkages. This latter method is particularly valuable when the labeling agents of interest are not readily incorporated into PCR generated GENE-TAGs or by enzymatically produced probes and reporters, such restricted label agents including but

not limited to, certain metallic or fluorescent compounds, latex or magnetic beads, and bioluminescent agents such as aequorin. Thus, Figure 15 depicts an alternative embodiment wherein a multiplicity of short labeled oligomers are aligned 3' and attached beforehand without requiring a secondary hybridization step. This design provides four-fold times four-fold times four-fold amplification, thereby binding 64 such oligos with fluorescent compounds or other labels attached. This embodiment of the present invention could thus overcome the current difficult of labeling standard probes with fluorescent compounds such as Cy3 and Cy5 that only allow 1% incorporation by PCR or reverse transcriptase. Thus, a standard single stranded probe about 600 bases long would only incorporate approximately six Cy3 or Cy5 flours while a short oligonucleotide based WRAP-PROBE linked to two such Multi-LINKER structures would be expected to provide 128 Cy3 or Cy5 flours per probe. This defined Multi-LINKER embodiment represents an alternative to the chaining TINKER-TAG reporter system shown in Figure 16, which is similarly employed to carry pre-labeled short oligonucleotides, but which does not provide such precise quantitation of signaling.

The invention further includes modified compositions and designs for improving the tethering of the probe and or the specificity of the detection. In one modification of the GAP-LOCK method especially suitable for microwell analyses, capture moieties are attached to the distal linker end of one probe; the dual probes are hybridized to the target strand and ligated together; the probe and target complex is fully denatured; and the ligated capture probe and reporters - joined via the ligation site - are captured and detected. See Figure 3A. This modification allows precise detection of mutations or base variations under liquid hybridization or near liquid hybridization conditions.

In other modifications, an additional component devised of partially overlapping oligonucleotides, known as a RING-TAIL unit, creates an incipient ring structure by being joined to GAP-LOCK or WRAP-PROBE components prior to hybridization to the target. Using GAP-LOCK components attached to two ends of the RING-TAIL unit, a composite RING-LOCK probe is hybridized to the target, the ends are ligated together to form a closed loop, and reporters are joined to linkers on the RING-TAIL unit. See Figure 17. A similar RING-TAIL unit can be combined with WRAP-PROBE components to create a composite WRAP-LOCK probe. This composite probe provides an incipient ring structure that loops around the target and is closed by hybridizing an overlapping oligonucleotide. See Figure 18.

The application of both of these probe methods creates a closed loop encircling the target strand, and at the same time, it supports the attachment of extended reporters such as arrays of GENE-TAG reporters. These modifications facilitate stringent washing or denaturation for more specific detection. The invention also includes a method for applying two such probes simultaneously to both the sense strand and the complementary antisense strand of a double-stranded DNA target in which the two probes have contrasting signaling. With this method, also known as the DOUBLE-LOCK design, the detection of a rare mutation or change can be confirmed by the detection of both signals at the same site. See Figure 19.

The practice of the present invention employs, unless otherwise indicated, conventional techniques of molecular biology, microbiology and recombinant DNA techniques, which are within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook, J. et al., *Molecular Cloning; A Laboratory Manual*, Second Edition (1989); *Oligonucleotide Synthesis* (M. J. Gait, ed., 1984); *Nucleic Acid Hybridization* (B.D. Hames & S.J. Higgins, eds., 1984) *A Practical Guide to Molecular Cloning* (B. Perbal, 1984); and the series *Methods in Enzymology* (Academic Press, Inc.).

The synthesis of the probe and reporter components of the present invention may be accomplished by conventional polymerase chain reaction (PCR) process. The protocol for PCR is set forth in Saiki et al., *Science* 230: 1350 (1985) and U.S. Pat. Nos. 4,683,195 and 4,683,202. A PCR adapter-linker method is set forth in Saunders et al. (1990); Johnson (1990) and PCT 90/00434. Another PCR method employing a mixture of primers is described in Meltzer et al., *Nature Genetics*, 1 (1): 24-28 (April 1992).

Probe and reporter components of the present invention are also synthesized by conventional means on a commercially available automated DNA synthesizer, e.g. an Applied Biosystems (Foster City, Calif.) model 380B, 392 or 394 DNA/RNA synthesizer. Preferably, phosphoramidite chemistry is employed according to, e.g., Beaucage et al., Tetrahedron, 48:2223-2311 (1992); Molko et al., U.S. Pat. No. 4,980,460; Koster et al., U.S. Pat. No. 4,725,677; Caruthers et al., U.S. Pat. Nos. 4,415,732; 4,458,066; and 4,973,679. In preferred embodiments of the present invention, the probe has a nuclease resistant backbone. Many types of modified oligonucleotides are available that confer nuclease resistance, e.g. phosphorothioate, phosphorodithioate, phosphoramidate. For phosphorothioates, see, e.g., Stec et al., U.S. Pat. No. 5,151,510; Hirschbein, U.S. Pat. No. 5,166,387; or Bergot, U.S. Pat.

No. 5,183,885. For phosphoramidates, see, e.g., Froehler et al., International application PCT/US90/03138. In some embodiments it may be desirable to employ P-chiral linkages, e.g., Stec et al, EPO 92301950.9.

In several embodiments of the present invention, modified oligonucleotides are synthesized with internal spacers, commonly composed of carbon chains, which separate different functional regions of the oligonucleotide. Typically, the 5' end of such a spacer oligonucleotide will serve as a probe end or a reporter linker end while the 3' end serves as a primer for PCR amplification. During PCR, the polymerase cannot jump across the spacer region of such an oligonucleotide and thus the probe or linker end beyond the spacer remains single stranded. Preferably, the spacer between the probe and primer ends is a phosphorus linking group, which includes, but is not limited to, phosphodiester, methyl or ethyl phosphonate, phosphorothioate, phosphoramidate, hydroxyurethane, carboxyaminoalkyl, or carboxyaminoalkyl-phosphate linkers. Generally, spacers derived from phosphoramidite precursors, such as the carbon chain Spacer Phosphoramidites C9 or C18 from Glen Research, Inc. (Sterling, Virginia), are preferred so that the modified oligonucleotides of the invention can be conveniently synthesized with commercial automated DNA synthesizers, e.g. Applied Biosystems, Inc. (Foster City, Calif.) model 394.

Spacer length may vary significantly depending on the nature of the probe and primer sequence. Preferably, spacer moieties are synthesized using conventional phosphoramidite and/or hydrogen phosphonate chemistries. Several phosphoramidite or hydrogen phosphonate monomers suitable for use in the present invention are set forth in Newton et al., Nucleic Acid Research, 21:1155-1162 (1993); Griffin et al., J. Am. Chem. Sot., 114:7976-7982 (1992) Jaschke et al., Tetrahedron Letters, 34:301-304 (1992); Ma et al, International application PCT/CA92/00423; Zon et al., International application PCT/US90/06630; Durand et al., Nucleic Acids Research, 18:6353-6359 (1990); and Salunkhe et al., J. Am. Chem. Soc., 114:8768-8772 (1992).

There is extensive background literature relating to the selection of hybridization conditions, labeling means, and the like, which is applicable to the principles and practice of the present invention. See, e.g. Wallace et al. Nucleic Acids Research 6:3543-3557 (1979); Crothers et al., J. Mol. Biol. 9:1-9 (1964); Gotoh, Adv. Biophys. 16:1-52 (1983) Wetruer, Critical Reviews in Biochemistry and Molecular Biology 26:227-259 (1991); Breslauer et al.,

Proc. Natl. Acad. Sci. 83:374-3750 (1986); Wolf et al., Nucleic Acids Research, 15:2911-2926 (1987); McGraw et al., Biotechniques, 8:674-678 (1990).

Conditions for annealing DNA probes to DNA or RNA targets are well known, e.g., *Nucleic Acid Hybridization, A Practical Approach* (B.D. Homes, eds.), IRL Press, Washington, D.C. (1985). In general, whether such annealing or hybridization takes place is influenced by the length of the probes and the test substances, the pH, the temperature, the concentration of mono- and divalent cations, the proportion of G and C nucleotides in the hybridizing region, the viscosity of the medium and the possible presence of denaturants. Such variables also influence the time required for hybridization. The preferred conditions will therefore depend upon the particular application. Such conditions, however, can be routinely determined without undue experimentation.

For GAP-LOCK probes, the preferred linking agent is a ligase, such as T4 DNA ligase, using well known procedures (Maniatis, T. in *Molecular Cloning*, Cold Spring Harbor Laboratory (1982)). Other DNA ligases are also suitable. T4 DNA ligase may also be used when the test substance is RNA [Engler, M.J. et al., *The Enzymes*, Vol. 15, pp. 16-17 (1982), Higgins, N. P. et al., *Methods in Enzymology*, Vol. 68, pp. 54-56 (1979)]. Ligases from thermophilic organisms, e.g. Tth DNA ligase, *Gene*, Vol. 109, pp. 1-11(1991), New England Biolabs, (Beverly, Mass.), and Ampligase, Epcentre Technologies, Inc. (Madison, Wisc.) are preferred, so that ligation at higher temperatures may be carried out, allowing the use of longer probes with increased specificity. The ligation, however, need not be an enzyme and, accordingly, the linking agent may be a chemical agent which will cause the probes to link unless there is a nucleotide base pair mismatching at the target nucleotide position. The invention will be described using T4 DNA ligase as the linking agent. This enzyme requires the presence of a phosphate group on the 5' end of one polynucleotide and a 3' OH group on the neighboring polynucleotide.

Ligation conditions are adjusted so that ligation will occur if there is a base pair match at the target nucleotide position and will not occur if there is a mismatch at that position. Assuming simultaneous annealing and ligation, the ligation may be performed at a temperature below the melting temperature of the annealed oligonucleotide probes. A suitable temperature for this purpose is about 5° C to about 30° C. below the melting temperature of the hybridized sequences. For ligation after hybridization, a suitable

temperature is about 37° C for T4 DNA ligase. Factors that determine whether or not mismatching at the target nucleotide position can be determined, as detected by ligation, include salt concentration and the amount of enzyme (ligase) used. Suitable salt concentrations range from 0 to 200 mM.

For covalent joining of probe components and reporter components of the present invention, or for joining probe and target complexes more firmly, the preferred cross linking agent is a bi- or tri-functional psoralen compound such as 4, 5', 8-trimethylpsoralen which intercalates the bases of hybridized DNA strands and causes covalent cross linking between them when treated with long wave ultraviolet light, preferably in the range of 312 to 360 nanometers. Site specific cross linking can also be facilitated by synthesizing an oligonucleotide probe component with a terminal psoralen molecule tethered by a carbon chain. Commercial reagents, such as C2 psoralen and C6 psoralen from Glen Research, Inc. (San Diego, Calif.), allow the termination of a synthetic oligonucleotide with an attached psoralen suitable for inducing crosslinking with double or triple strand configurations, respectively, using standard phosphoramidite chemistry on a automated DNA synthesizer, e.g. Applied Biosystems, Inc. (Foster City, Calif.) model 394. The durability of complementary hybridization between probe and reporter components may also be increased by employing artificial nucleotides; e.g. pdC-CE, pdU-CE, 5-Me-dC, Glen Research, Inc. (Sterling, Virginia), which can significantly raise melt temperature (T_m) by several degrees, and can diminish non-specific binding of these components.

The reporter molecules of this invention can be labeled during PCR amplification in the presence of appropriately modified dNTPs, or they can be labeled after completion of the PCR reaction by chemical or enzymatic modification of the PCR products. When the reporters are constructed of synthetic oligonucleotides, they can be labeled directly or indirectly by incorporating modified bases that either carry labeling agents or that provide chemical or immunological means for the attachment of labeling agents. Alternatively, such reporters may contain secondary linkers for binding short oligonucleotides that are conjugated to labeling agents - usually at one end.

Any of the various labeling techniques, direct or indirect, may be used to label reporters, including but not limited to fluorescent chemicals, radioactive materials, chemical haptens, or enzymatic modifiers. More than one label can be used. Preferred modified

dNTPs include but are not limited to biotin-16-dUTP; digoxigenin-11-dUTP; biotin derivatives of dATP; fluoresceinated-dUTP; rhodamine labeled derivatives of dUTP or dCTP; hydroxy coumarin-labeled derivatives of dUTP; resorufin-11-2'-dUTP, and thiol or amine modified dNTPs, e.g. Amino-Modifier C6-dT, Glen Research, Inc. (Sterling, Virginia). Other potential labels that may be attached or conjugated to reporter components include but are not limited to: (1) gold and silver particles; e.g. monomaleimido Nanogold, LI Silver, etc., Nanoprobes, Inc., (Stony Brook, NewYork); Colloidal Gold, Sigma Chemical Co. (Saint Louis, Missouri); (2) chemiluminescent or bioluminescent molecules such as aequorin, e.g. Aqualite, Sealite Sciences, Inc., (Norcross, Georgia); and (3) agents which can provide Raman spectrometry signaling such as DNA and histological dyes; e.g. Methyl green, Cresyl fast violet, Acridine orange, Ponceus S, Malachite green oxalate, Luxol fast blue, Cresyl violet acetate and Bromophenol blue; double and or triple bonded chemical labels; e.g. Chloracetonitrile, Propargyl chloride, 3'Methoxybenzyl chloride and alpha Bromo p-tolunitrile, Aldrich Chemical Company, Inc. (Milwaukee, Wisc.); and propyne or methyl modified phosphoramidite nucleosides; e.g. pdC-CE, pdU-CE, 5-Me-dC, Glen Research, Inc. (Sterling, Virginia).

Gold and silver labeling is of particular interest for the TINKER-TAG reporter system wherein gold particles, e.g. monomaleimido Nanogold, Nanoprobes, Inc., (Stony Brook, New York), can be conjugated to oligonucleotides that bind to the side arms of the reporters and then silver ions, e.g. LI Silver, Nanoprobes, Inc., (Stony Brook, New York), can be added to precipitate large clusters of silver of 30 to 100 nm around the gold seeds forming dense masses suitable for light microscopy visualization. The size of the cluster may vary with the size of the gold particle. This reporter variation, also known as the GOLD-TAG method, provides the advantage of avoiding the safety, expense and equipment requirements of radioactive or fluorescent labeling. See Figure 20.

Bioluminescent immunoassays using the photoprotein aequorin have attained sensitivity in the zeptomole range (10^{-21} mol) which is at least 10^{6} times more sensitive than chemiluminescent enzyme immunoassays in widespread use. The bioengineered aequorin contains three calcium binding sites and, upon addition of calcium ions, undergoes a

conformational change that catalyzes the oxidation of a bound coelenterazine producing a brief flash of blue light that peaks in 0.3 seconds, centered at 469 nm.

For such experiments, biotinylated PCR amplicons of the target nucleotide sequence are captured in streptavidin-coated microtiter plate wells, and detected with probes and reporters labeled with digoxigenin.e.g., dig-11-dUTP. These digoxygenin haptens then bind a stable, covalent conjugate between the aequorin photoprotein and the sheep anti-digoxigenin Fab fragment. When stimulated by calcium, the amount of light produced is directly proportional to the amount of digoxigenin detected. Alternatively, aequorin molecules can be directly conjugated to the 5' end of simple oligonucleotides that can be secondarily linked to probe and reporter units of the present invention such as the TINKER-TAG constructs. The use of said aequorin conjugated oligonucleotides as labeling for TINKER-TAG reporters is expected to enhance bioluminescent signal detection in microwell formats while maintaining the advantages over competing technologies in sensitivity, low background, stability, and simplicity of assay design. See Figure 16.

The staining intensity achieved using the reporters may be amplified with a variety of systems, including but not limited to fluorochrome conjugated avidin and/or labeled antibodies. Similarly, other known detection schemes such as labeling of probe molecules with enzymes, sulfur or mercury may be applied in order to suit special experimental conditions. DNA can be counterstained with DNA-specific dyes, including but not limited to DAPI, that fluoresce in different wavelength intervals dependent on the selected scheme for visualization of bound probe molecules.

Methods for introducing oligonucleotide functionalizing reagents or to introduce one or more sulfhydryl, amino or hydroxyl moieties into the reporter sequence are described in U.S. Pat. No. 4,914,210. Such modified nucleotides can provide multiple signaling sites by incorporating them along the length of the reporter molecule or at the ends of attached oligonucleotides. A 5' phosphate group can be introduced as a radioisotope by using polynucleotide kinase and gamma ^{32}P-ATP to provide a reporter group. Biotin can be added to the 5' end by reacting an aminothymidine residue, or a 6-amino hexyl residue, introduced during synthesis, with an N-hydroxysuccinimide ester of biotin. Labels at the 3' terminus

may employ polynucleotide terminal transferase to add the desired moiety, such as for example, cordycepin ^{35}S-dATP, and biotinylated dUTP.

The present invention generally combines WRAP-PROBE or GAP-LOCK probe designs, and various manifestations thereof, with the GENE-TAG or COLOR-TAG signal amplificaton systems. Alternatively, these probe designs could be employed in combination with other commercial probe and signaling systems such as the dendrimers of Polyprobe, Inc. (Media, Penn.) [U.S. Pat. No. 5,487,973] and the branch DNA (bDNA) components of Chiron Corp. (Emeryville, Calif.) [U.S. patent 5,124,246]. See Figure 21A. The Chiron products are designed to detect a gene by creating a series of unique oligonucleotide probes which bind in part to the target sequences and which have singular generic tails that bind to the bDNA structures. Because this system relies on simple hybridization to hold down the synthetic bDNA signaling structures, effective gene detection requires multiple probes targeting multiple locations along a gene. However, the substitution of a GAP-LOCK or WRAP-PROBE element instead of a simple tailed oligonucleotide probe would provide a wrapped structural configuration that greatly exceeds the forces of hybridization, and which would support, at the least, two times the bDNA signal structure of their current probes. Similarly, the dendrimers of Polyprobe also rely on simple hybridization to bind the large signaling structure to the target - generally by binding the target sequence with one of the multiple single stranded arms that protrude from the outer layer. Again, the WRAP-PROBE or GAP-LOCK probes could be employed very effectively, in combination with these dendrimers, to either bind larger dendrimers or to bind them more durably and or more specifically to the target.

The probes of the present invention can be employed as diagnostic probes to detect the presence of one or more target polynucleotides in a wide range of samples, including diagnostic applications of nucleic acid probes in human diagnostics, forensics, and genetic analysis. See, e.g., Caskey, Science 236:1223-1228 (1987); Landegren et al. Science, 242:229 237 (1988); and Arnheim et al., Ann. Rev. Biochem., 61:131-156 (1992). Other uses for the DNA molecular diagnostic devices of the present invention include environmental samples, e.g. from public water supplies, samples from foodstuffs, and from other biological samples, such as blood, saliva, lung sputum, semen, buccal smears, urine or fecal waste, cell

biopsies, amniotic fluid, tissue homogenates of plants or animals, or of human patients, and the like.

These probes and reporter systems can be readily employed in a variety of membrane formats such as dot blots, slot blots, Southern and Northern blots, and expression microarrays; in gels such as agar or polyacrylamide; in a variety of in situ formats such as for FISH (fluorescent in situ hybridization) to map genes on chromosomes or for in situ detection of RNA transcripts or genes in sectioned tissue and/or tissue microarrays; in microwell plates to detect infectious organisms or unbound DNA fragments extracted from bodily fluids or wastes; and in various solid substrate chip formats to detect genes, mutations or mRNA including, but not limited to, oligonucleotide microarrays, cDNA microarrays, and molecular detection chips employing flourescence, optical interferometry, Raman spectrometry or semi-conductor electronics.

Example 1

GAP-LOCK Probe Method:

The present example demonstrates the application of the GAP-LOCK dual probe mechanism to detect a critical Mycobacterium tuberculosis (MTB) sequence which is diagnostic of that infectious agent. Special components for the two GAP-LOCK probe units were made on an ABI 394 automated synthesizer by synthesizing three modified oligomers with two C9 internal spacers (nine carbon phosphoramidite products made by Glen Research, Sterling, Virginia). (In all sequence descriptions below a C9 spacer is shown as "9" to avoid confusion with cytidine bases [C].) Of these modified oligomers, two of them had M13 forward sequences on their 3' end to serve as a primer for PCR amplification. One of them, called here the 5' First Probe Oligo (FP) (SEQ ID NO: 1,2), has one-half the MTB target sequences on the 5' end. The other one, called the Reversing Linker Oligomer (SEQ ID NO: 6,7), has a set of generic linker sequences on that end. The third modified oligomer, the 3' Reverse Probe Oligo (RP) (SEQ ID NO: 4,5), has linker sequences on the 5' end that match the linker sequences on the Reverse Linker Oligo, and the 3' end had the other half of the target sequences. The use of C9 spacers is optional for the 3' Reverse Probe Oligo. Two such oligomers were then employed in PCR reactions to create long reporter components, using arbitrary M13 plasmid DNA inserts 300 or 800 base pairs long as templates for amplification.

See Fig. 2A. During PCR, the M13 forward sequences on the 3' end of the modified oligomers served as the first primer. A simple oligonucleotide of M13 reverse sequences (SEQ ID NO: 147) served as the second primer. ^{32}P-labeled thymidine bases were incorporated during PCR on a ratio of 1 labeled base to 3 unlabeled thymidine bases. Thirty cycles of PCR were run and the reporter products were purified by microcentrifugation. One product, constituting the First Probe, had a 5' head end containing sequences complementary to half the MTB target site and the 5' end was phosphorylated. The second PCR reporter product is incomplete since the 5' head end has generic linker sequences that only serve to reverse the 5'-3' orientation. The Reverse Probe was created by annealing that second PCR product with the 3' Reverse Probe Oligo for 20 minutes at 42^{o}C in PCR buffer. The resulting composite Reverse Probe has a 3' head end containing the tandem second half of the target sequences.

The annealed oligos were crosslinked together with psoralen plus UV, using 3 µg/µl psoralen (4,5',8-trimethylpsoralen) for 15 minutes, and secondly exposing the samples to 15 minutes of long wave UV (312nm) in clear microfuge tubes while placed on a UV light box. Psoralen preferentially intercalates and crosslinks at 5' TA sites. Additional gel shift experiments were conducted with crosslinked probe components wherein the linker sequence contained only one 5' TA site vs. five 5' TA sites. Such crosslinking appears efficient since it was found that both complexes remained intact and were equally resistant to 10 minutes of 0.05 M NaOH treatment.

These GAP-LOCK probes were tested on DNA dot blots by standard methods using ^{32}P-labeling and nylon membrane supports. The primary target DNA was a known amplified 600 bp fragment of the 16s ribosomal gene of Mycobacterium tuberculosis. Negative control DNA samples were amplified from a similar-sized but sequence-unrelated fragment of the bombesin receptor gene. In all dot blot experiments, solutions of the target DNAs were applied in 1 µl aliquots to nylon membranes in multiple 40 ng dots or in a series of 50% dilutions starting with 40 ng of DNA in the first dot. The DNA was denatured with 0.5 M NaOH and neutralized with 5xSSC and 0.1% SDS solution. The denatured target DNAs was crosslinked to the membranes by exposing them to 2000 mJ of 253 nm UV in a UV crosslinker. Hybridizations were performed at 42^{o}C in a rotary apparatus running at

approximately 10 rotations per min. The membranes were transferred to individual roller tubes, pretreated in the apparatus for 2 hours with hybridization buffer (5xSSC, 1% SDS, 5xDenhart's, 1xBackground Quencher) before the probe sample was added and hybridization was continued. Unlike standard DNA probes, the probe and reporter units were not denatured. After hybridization, the blots were washed for 30 min in 5xSSC/0.5% SDS at 42°C followed by 2 washes in 0.5xSSC/0.5% SDS for 20 min each at 55°C. All dot blot labeling was detected with a phosphor imager.

Eight strips of nylon membranes were loaded with paired dots of both TB and bombesin receptor target DNAs that were denatured and crosslinked to the nylon membrane. After the dot blots were pre-treated with hybridization buffer at 42°C, the First Probe (FP) and the Reverse Probe (RP) with reporters attached were added for hybridization overnight (14 to 18 hrs). One set of four strips were treated with probes with reporter templates of about 300 bp while the other set of four strips were treated with probes with reporter templates of about 800 bp. The first strip in each set of four used only the First Probe (FP). The second strip in each set of four used only the Reverse Probe (RP). The third and fourth strip in each set used both the First Probe and the Reverse Probe (F/R), however, only the fourth strip was treated for ligation (F/R +Lig).

These membranes were transferred to small 30 ml plastic petri dishes and treated with T4 kinase at 37°C for 30 min, to phosphorylate the 5' half-probe end, and with T4 ligase at 37°C for 30 min. After the ligation step, the strips were read with the phosphor imager. The strips were then treated for 5 minutes with dilute alkali (0.05 M NaOH), neutralized, and rinsed to denature and release any unligated probe and reporter units. These washed, second half-blot strips were then read on the phosphor imager and compared to the first half-blot strips.

The selective retention of labeling after ligation shows that joining the dual probe units into a single GAP-LOCK probe complex confers a more stable wrapped configuration. See Figure 2C. The resulting ligated probe complex encircles the target at mid-point and the reporters extend from both sides thereby counterbalancing one another. Consequently, longer reporters can confer greater stability than short reporters. Therefore, ligation essentially locks the probe to the target making it resistant to removal by denaturation or stringent washing,

and this mechanism can be employed to detect and discriminate single base mutations or variations.

GAP-LOCK Probe and Attached reporter TAGs for MTB Detection:

A. 5' First Probe Oligomer: (SEQ ID NO: 1,2)
| upstream target region spacers M13 forward primer |
5'ACCACAAGACATGCATCCCG-99-CCAGGGTTTTCCCAGTCACGAC

B. Second M13R Primer: (SEQ ID NO: 3) 5' GAGCGGATAACAATTTCACACAGG

First Probe Fabrication: Synthesize Oligomers A and B. Using Oligomers A and B as primers, run a PCR reaction using an M13 DNA template (300 or 800 bp in length) to produce a long double stranded reporter with a single stranded half-probe on one end.

C. 3' Reverse Probe Oligomer: (SEQ ID NO: 4,5)
| generic linker to 5' end of D spacers downstream target region |
5' GAGCCTGGCTCACCCTAGGTCCAG-99-CCCACACCGCTAAAGCGCTTTCC

D. Reversing Linker Oligomer: (SEQ ID NO: 6,7)
| generic linker to 5' end of C spacers M13 forward primer |
5'CTGGACCTAGGGTGAGCCAGGCTC-99-CCAGGGTTTTCCCAGTCACGAC

E. Second M13R Primer: (SEQ ID NO: 3) 5' GAGCGGATAACAATTTCACACAGG

Reverse Probe Fabrication: Synthesize Oligomers D, C and E. Using Oligomers D and E as primers and a M13 DNA template of 300 or 800 bp, run a PCR reaction to produce a long double stranded generic reporter TAG with a single stranded linker on one end. Oligomer C is then joined to the generic TAG unit via the generic linker sequences thereby adding a single stranded half-probe with a 3' end. The Oligomer C can be crosslinked to the generic reporter TAG unit using free psoralen plus UV light.

Application: Prepared eight strips of dot blots with 4 dots of 40 ng target DNA per strip. After hybridization (16 hrs) and first wash, strips were cut in half, one half read on a phosphor imager immediately, the other treated with 0.05 M NaOH for 5 min, neutralized, washed, and read again on a phosphor imager.

All probe combinations with short reporters proved sensitive to NaOH removal even with ligation since the short reporters can unwind from the target site. See Figure 2C. However, with long reporters attached to the probes, the ligated dual probes are preferentially retained despite alkali denaturation of the probe from the target. This advantage occurs because the long reporter tails, extending from both sides of the central wrapped probe configuration, create a probe/reporter complex that is more difficult to unwind and wash away.

Example 2

WRAP-PROBE Method and Chained Reporter GENE-TAG Method:

Equimolor portions of the two probe subunits (SEQ ID NO: 8 and SEQ ID NO: 9) were mixed together in a clear, thin-walled microfuge tube containing PCR buffer solution, and the mixture is incubated at 42°C for 10 minutes to hybridize the two oligomers together. The annealed oligomers were then covalently crosslinked with psoralen and UV by: 1) adding 2 ml of 3 mg/ml 4,5',8-trimethylpsoralen for 10 minutes and 2) placing the tube on a light box emitting 312 nm UV for 20 minutes while covered with aluminum foil. In other experiments, a 15 to 20 minute exposure to a 15 Watt blacklite bulb (360 nm) at 1 to 2 cm was effective. The resulting joined probe complex was thus target specific at mid-portion, but with dual 5' tails that can link to reporter units.

~~WRAP-PROBE to detect MTB 16sRNA sequences~~

A. Target-Specific Oligomer: (SEQ ID NO: 8)
|5' linker to reporter 3T spacer s target specific region 3T spacer s link to 3' end of B |
GTAGCCTAGCTACCCCTAGGTCTAGTTTACCACAAGACATGCATCCCGTTTGTAG
ATAGGTAG

B. Generic Overlap Oligomer: (SEQ ID NO: 9)
| linker to reporter link to 3' end of A |
5' GTAGCCTAGCTACCCCTAGGTCTAG-CTACCTATCTAC

5'GTAGCCTAGCTACCCCTAGGTCTAGTTTACCACAAGACATGCATCCCGTTT-
GTAGATAGGTAG
\ \ \ <--

TA crosslink sites

CATCTATCCATC----

GATCTGGATCCCCATCGATCCGATG

Probe Assembly: Sequence A was joined to Sequence B via complementary 2nd linker sequences and then they were covalently crosslinked together via three internal TA sites using free psoralen plus UV treatment.

Example 3

Preparation of GENE-TAG Reporters: GENE-TAGS and Terminal TAGS

In each PCR reaction, ten femtograms of template DNA from any phage, e.g., M13, were incubated with 0.5 U Taq polymerase, 100 mM dNTP, 20 mM of labeled dUTP or dATP, standard PCR buffer with 4.0 mM magnesium chloride, and 0.2 mM of each primer or modified oligomer in a 100 ml volume. In most experiments, the signaling nucleotide was labeled with digoxigenin or ^{32}P. The labeling ratio was 1:3 (labeled vs. unlabeled). Other labeling haptens, such as biotin, fluorescein (FITC) or dinitrophenol are also suitable. Thirty to forty PCR thermal cycles were run at a melting temperature of 94°C for 30 sec, annealing at 50 to 55°C for 30 sec, and extension at 72°C for 45 sec to 1.5 min. Products were tested by ethidium bromide electrophoresis. Gel purification of the GENE-TAG reporters was not required although unincorporated nucleotides, primers and reaction byproducts were removed with microcentrifuge filtration.

GENE-TAGs and Terminal TAGs were pre-formed as reporter chains of statistically defined length by premixing them together in defined proportions under annealing conditions. If a 3:1 mix of GENE-TAGs to Terminal TAGs is made, most chains will consist of 4 units, many will contain 2 or 5 units, and only a few will have 1 unit or 6 or more units. In most experiments, GENE-TAGs/Terminal TAG pre-mixes ranging from 1:1 to 8:1 were used. In some experiments, only Terminal TAGs or only GENE-TAGs were used. The latter approach allows the formation of chains of GENE-TAGs of indeterminate length and signaling was generally reduced in such samples relative to samples where the chain was intentionally ended with a GENE-TAG/Terminal-TAG ratio of 3:1 or better. Alternatively, unjoined GENE-TAGs may be added to the hybridization solution after probe hybridization,

and then Terminal TAGs or oligonucleotide TERMINATORS (e.g., SEQ ID NO: 17) may be added after a period of time to stop extension of the reporter chain. With the latter approach, optimization of reporter efficiency is determined empirically. The examples below illustrate some oligomers containing Psoralen C2 molecules added to their 5' end so that chains of GENE-TAGs could be crosslinked together and to the probe to which they are joined. This crosslink-ready modification is optional and is generally not needed.

GENE-TAGs for MTB Wrap Probe:
A. Proximal Linker Oligomer: (SEQ ID NO: 10,11)
| Psoralen c2- linker spacers M13 reverse primer |
5'Psor.-TAGACCTAGGGGTAGCTAGGCTAC-99-
GAGCGGATAACAATTTCACACAGG

B. Distal Linker Oligomer: (SEQ ID NO: 12,13)
| linker spacers M13 forward primer |
5' GTAGCCTAGCTACCCCTAGGTCTAG-99-CCAGGGTTTCCCAGTCACGAC

GENE-TAG fabrication: Oligomers A and B serve as primers in a PCR reaction using a M13 DNA template 800 bp long. ^{32}P-labeled dTTP bases are incorporated during PCR producing a long double stranded reporter with single stranded linkers on each end.

Terminal TAG for MTB Wrap Probe:
C. Proximal Linker Oligomer: (SEQ ID NO: 14,15)
| Psoralen c2- linker spacers M13 reverse primer |
5'Psor.-TAGACCTAGGGGTAGCTAGGCTAC-99-
GAGCGGATAACAATTTCACACAGG
D. Second M13 F Primer: (SEQ ID NO: 16) 5' CCAGGGTTTCCCAGTCACGAC

Terminal TAG fabrication: Oligomers C and D serve as primers in a PCR reaction using an M13 DNA template 300 bp long. ^{32}P-labeled dTTP bases are incorporated during PCR producing a long double stranded reporter with a single stranded linker on one end. In some experiments, (Figure 5A2) the Terminal TAGs were not labeled and were just used to end a chain of GENE-TAGs.

Alternate: TERMINATOR: (used instead of Terminal TAG):

TAGACCTAGGGGTAGCTAGGCTAC (SEQ ID NO: 17)

Example 4

WRAP-PROBE Detection with nylon membrane DNA dot blots:

The ability of ^{32}P-labeled WRAP-PROBEs to specifically hybridize and label nucleic acids were tested with serial dot blots of MTB target DNA using the same methods employed in Example 1 above with respect to: preparing dot blots of target and control DNAs on nylon membrane supports; prehybridizing treatment; hybridizing probes and reporters in a rotary apparatus at 42°C; the composition and use of hybridization and wash buffers; and reading labeling with a phosphor imager.

Concentrated samples of the WRAP-PROBEs units were added to the roller tubes and hybridization was performed either overnight for 14 hours (Fig. 5A [A1]) or for 4 hours (Fig. 5A [A2]). The solution was then poured off and replaced with fresh pre-heated hybridization buffer containing pre-mixed and preannealed reporters. Hybridization was then continued for 2 to 4 hours. Blots were washed for 30 min in 5xSSC/0.5% SDS at 42°C followed by 2 washes in 0.5xSSC/0.5% SDS for 20 min each at 55°C before reading with a phosphor imager.

The specific comparisons in Figure 5A. [A1] and [A2] were to determine the relative detection of WRAP-PROBEs with reporter chains of increased GENE-TAGs (or G) vs. Terminal TAG (or T) ratios. In other experiments it was established that the probes were specific for MTB DNA detection and very little labeling of the bombesin receptor control DNA could be detected. Other experiments also demonstrated improved labeling for WRAP-PROBEs combined with these GENE-TAG reporter chains vs. GAP-LOCK First Probes combined with these same GENE-TAG reporter chains. In the first experiment illustrated here, Fig. 5A. [A1], the TAGs were mixed in the following ratios of GENE-TAGs to Terminal TAGs: 1:1, 2:1, 8:1. The WRAP-PROBE was applied for 14 hrs, the TAG mixture was added for 4 hours, and after washing, the samples were read with a phosphor imager. In the second experiment, Fig. 5A. [A2], the TAG ratios were changed to 1:1, 2:1 and 4:1 and ^{32}P was only incorporated into the GENE-TAG components. The unlabeled Terminal TAGs

used a 300 bp template. Probe hybridization was shortened to 4 hours and TAG hybridization to 2 hours.

Example 5

WRAP-PROBE Method and TINKER-TAG Reporter Method:

I. Probe Example: WRAP-PROBE to detect MTB similar to Example 2 above:

A. Target-Specific Oligomer: (SEQ ID NO: 18)
| linker to reporter 3t spacer s target specific region 3t spacer s link to B |
5' GTAGCCTAGCTACCCCTAGGTCTAG-TTT-ACCACAAGACATGCATCCCG-TTT---

GTAGATAGGTAG

B. Generic Overlap Oligomer: (SEQ ID NO: 19)
| linker to reporter link to A |
5' GTAGCCTAGCTACCCCTAGGTCTAG - CTACCTATCTAC

Using methods described in Examples 1-4, synthesize A and B oligonucleotides. Hybridize and crosslink A and B subunits together to form WRAP-PROBE.

5' Linker to reporter 3t spacer target region 3t spacer link to B
GTAGCCTAGCTACCCCTAGGTCTAGTTTACCACAAGACATGCATCCCGTTT-
GTAGATAGGTAG
\ \ \ <-- TA
crosslink sites
CATCTATCCATC---

GATCTGGATCCCCATCGATCCGATG
link to A Linker to reporter 5'

II. Reporter Example: TINKER-TAG Method:

A. Proximal Subunit: (SEQ ID NO: 20)
| 1st link to probe/prior unit - overlap - link to Label Subunit |
5'CTAGACCTAGGGGTAGCTAGGCTAC-ATACGATACTAG-
GGCATAACATAGGCTTACCA

B. Distal Subunit: (SEQ ID NO: 21)
| 1st link to next proximal subunit - overlap - link to Label Subunit |

5'GTAGCCTAGCTACCCCTAGGTCTAG-CTAGTATCGTAT-
GGCATAACATAGGCTTACCA

C. Label Subunit: (SEQ ID NO: 22)
5' LABEL MOIETY - TGGTAAGCCTATGTTATGCC

D. Terminator: (SEQ ID NO: 23) 5' CTAGACCTAGGGGTAGCTAGGCTAC

Using methods described in Examples 1-4, synthesize A, B, C and D oligonucleotides. Hybridize and crosslink multiple A and B subunits together to form linear TINKER-TAG chains, length limited by the addition of a small proportion of Terminators (D subunit). Depending on the signaling characteristics of the label employed, the Label Subunits (C) are added to the second linker side arms at the same time or after the probe and TINKER TAG units are complexed to the target site. See Figure 6. Labeling molecules include but are not limited to aequorin, enzymatic catalysts, fluorochromes, dyes and metallic particles.

<u>TINKER-TAG Subunit with side arms to link to Label Subunits:</u>

```
                                                      FREE SIDE ARM
                                                    --GGCATAACATAGGCTTACCA 3'
BINDS TO PROBE OR  PROXIMAL TAG                          /
 5' CTAGACCTAGGGGTAGCTAGGCTAC-ATACGATACTAG-
                                                                      -TATGCTATGATC-
GATCTGGATCCCCATCGATCCG ATG 5'
                                            /                  BINDS TO NEXT TAG
     3'  ACCATTCGGATACAATACGG--
        FREE SIDE ARM
```

<u>TINKER-TAG Subunit with Label Subunits attached to side arms:</u>

```
                                                           CCGTATTGTATCCGAATGGT -
LABEL 5'
                                                       --GGCATAACATAGGCTTACCA
                                                       /
   5' CTAGACCTAGGGGTAGCTAGGCTAC-ATACGATACTAG-
                                                                  -    TATGCTATGATC-
GATCTGGATCCCCATCGATCCG ATG  5'
                                              /
          ACCATTCGGATACAATACGG--
5'LABEL - TGGTAAGCCTATGTTATGCC
```

Using target preparation and hybridization protocols described in Examples 1-4, hybridize probe to target, wash, hybridize TINKER-TAG arrays to probe, wash, hybridize Label Subunits to probe and TINKER-TAG complex if not pre-attached. Detect labeling to indicate target.

Example 6

RING-LOCK Probe:

Probe Components: (targeting sense strand of MTB 16s RNA sequences)
A. 5' First Probe Subunit: (SEQ ID NO: 24)
| upstream target region 3T spacers link to 3' end of C |
5' GGTCCTATCCGGTATTAGA-TTT-CTAGGGTTACCATA

B. 3' Reverse Probe Subunit: (SEQ ID NO: 25)
| link to 5' end of E 3T spacers downstream target region |
5' GCTACTTAGCATAC-TTT-ACCACAAGACATGCATCCCGT

RING-TAIL Components:
C. Forward Ring Subunit: (SEQ ID NO: 26,27)
| generic link to reporters 3Ts overlap region 3c9s link to 3' end of A |
5'TAGACCTAGGGGTAGCTAGGCTAC-TTT-ATAAGTACGTAGC-999-TATGGTAACCCTAG

D. Reverse Ring Subunit: (SEQ ID NO: 28,29)
| generic link to reporters 3Ts overlap region 2c9s link to 3' end of E |
5'TAGACCTAGGGGTAGCTAGGCTAC-TTT-GCTACGTACTTAT-99-CGTACTTACTAGCA

E. Reversing Oligonucleotide: (SEQ ID NO: 30)
| link to 5' end of B link to 3' end of D |
5' GTATGCTAAGTAGC-TGCTAGTAAGTACG

Using methods described in Examples 1-4, synthesize oligonucleotide subunits A, B, C, D and E. RING-TAIL components may be prepared in bulk. Combine and covalently join equimolor proportions of A to E by standard hybridization and crosslink methods to form RING-LOCK unit. Using methods described in Examples 2-5, manufacture GENE-TAG

reporters or other reporters with linkers complementary to the RING-TAIL unit. See Figure 17.

Using methods described in Examples 1-5, prepare target sample, hybridize and ligate RING-LOCK unit to target, denature or wash stringently, hybridize reporters and detect target mutation or sequence variation.

Example 7

WRAP-LOCK Probe Method:

WRAP-PROBE Component: to detect MTB similar to Example 2 above:
A. Target-Specific Oligomer: (SEQ ID NO: 31)
| link to 5' end of D 3t spacer s target specific region link to 3' end of B |
5'GGTAGTAGCTAGCACCTAGGCACCTTA-TTT-ACCACAAGACATGCATCCCG-TTT--
--CTAGGGTTACCATA

RING-TAIL Components:
B. Forward Ring Subunit: (SEQ ID NO: 32,33)
| link to reporters 3Ts overlap 3c9s link to 3' end of A |
5'TAGACCTAGGGGTAGCTAGGCTAC-TTT-ATAAGTACGTAGC-999-
TATGGTAACCCTAG

C. Reverse Ring Subunit: (SEQ ID NO: 34,35)
| link to reporters 3Ts overlap 2c9s link to 3' end of D |
5'TAGACCTAGGGGTAGCTAGGCTAC-TTT-GCTACGTACTTAT-99--
--
CGTTACGCTAACCTGCTATCTACCACT

LOCK Component:
D. Overlap Oligomer: (SEQ ID NO: 36)
| link to 5' end of A link to 3' end of C |
5'TAAGGTGCCTAGGTGCTAGCTACTACC-
AGTGGTAGATAGCAGGTTAGCGTAACG

Using methods described in Examples 1-4, synthesize oligonucleotide subunits A, B, C and D. RING-TAIL components (B and C) and LOCK component (D) may be prepared in bulk. Combine and covalently join equimolor proportions of A, B and C components by standard hybridization and crosslink methods to form WRAP-LOCK unit. Using methods

described in Examples 2-5, manufacture GENE-TAG reporters or other reporters with linkers complementary to the RING-TAIL unit. See Figure 18.

Using methods described in Examples 1-5, prepare target sample, hybridize WRAP-LOCK unit to target. Hybridize Overlapping LOCK component to close incipient circle of WRAP-PROBE and RING-TAIL unit complexed to target. Complex of probe and LOCK component may be treated with psoralen plus UV to form covalently closed circle. Wash stringently, hybridize reporters and detect target sequence.

Example 8

DOUBLE-LOCK Probe Method:

First Probe Components: (targeting sense strand of MTB 16s RNA sequences)
A. 5' Probe Subunit: (SEQ ID NO: 37)
| upstream target region 3T spacers link to 3' end of C |
5' Phos. GGTCCTATCCGGTATTAGA-TTT-CTAGGGTTACCATA

B. 3' Probe Subunit: (SEQ ID NO: 38)
| link to 5' end of E 3T spacers downstream target region |
5' GCTACTTAGCATAC-TTT-ACCACAAGACATGCATCCCGT

Type 1 RING-TAIL Components:
C. Forward Ring Subunit: (SEQ ID NO: 39,40)
| link to reporters 3Ts overlap region 3c9s link to 3' end of A |
5'TAGACCTAGGGGTAGCTAGGCTAC-TTT-ATAAGTACGTAGC-c9c9c9-TATGGTAACCCTAG

D. Reverse Ring Subunit: (SEQ ID NO: 41,42)
| link to reporters 3Ts overlap region 2c9s link to 3' end of E |
5'TAGACCTAGGGGTAGCTAGGCTAC-TTT-GCTACGTACTTAT-99-CGTACTTACTAGCA

E. Reversing Oligonucleotide: (SEQ ID NO: 43)
| link to 5' end of B link to 3' end of D |
5' GTATGCTAAGTAGC-TGCTAGTAAGTACG

Second Probe Components: (targeting antisense strand of MTB 16s RNA sequences)
F. 5' Probe Subunit: (SEQ ID NO: 44)
| downstream target region 3T spacers link to 3' end of H |
5' Phos.-ACGGGATGCATGTCTTGTGGT-TTT-CTAGGGTTACCATA

G. 3' Probe Subunit: (SEQ ID NO: 45)
| link to 5' end of J 3T spacers upstream target region |
5' GCTACTTAGCATAC-TTT-TCTAATACCGGATAGGACC

Type 2 RING-TAIL Components:
H. Forward Ring Subunit: (SEQ ID NO: 46,47)
| link to reporter type 2 3Ts overlap region 3c9s link to 5' end of F |
5'GATACCGTAGCTACGCGTACTACG-TTT-ATAAGTACGTAGC-999-TATGGTAACCCTAG

I. Reverse Ring Subunit: (SEQ ID NO: 48,49)
...| link to reporter type 2 3Ts overlap region 2c9s link to 3' end of J |
5'GATACCGTAGCTACGCGTACTACG-TTT-GCTACGTACTTAT-99-CGTACTTACTAGCA

J. Reversing Oligonucleotide: (SEQ ID NO: 50)
| link to 5' end of G link to 3' end of I |
5' GTATGCTAAGTAGC-TGCTAGTAAGTACG

Using methods described in Examples 1-6, synthesize oligonucleotide subunits A to J. Type 1 and Type 2 RING-TAIL components are prepared in bulk. Combine and covalently join equimolor proportions of A to E by standard hybridization and crosslink methods to form Type 1 sense strand RING-LOCK unit. Separately, combine and covalently join equimolor proportions of F to J by standard hybridization and crosslink methods to form Type 2 antisense strand RING-LOCK unit. Using methods described in Examples 2-5, manufacture two Types of GENE-TAG reporters or other reporters with linkers complementary to either the Type 1 or Type 2 RING-TAIL unit. See Figure 19.

Using methods described in Examples 1-6, prepare denatured target sample, hybridize and ligate Type 1 and Type 2 reporters, and detect target mutation or sequence variation with detection preference given to sites containing both Type 1 and Type 2 signaling.

Example 9

GAP-LOCK First Probe Method with Aequorin Bioluminescence:

Experiments (Figure 20) demonstrate highly sensitive detection when GAP-LOCK first probe constructs were employed in a microtiter format with MTB target DNA using aequorin conjugated anti-digoxygenin detection of digoxygenin labeling. Aequorin (trade name Aqualite) is a bioluminescent commercial detection product developed by Sealite Sciences, Inc. (Norcross, Georgia).

Bioluminescent detection of MTB target DNA: Biotinylated PCR target product was denatured for 5 min (1 M NaOH, 200 mM EDTA), neutralized (0.15 M Na2HPO4, pH 6.0), and added to streptavidin-coated microtiterplates, containing 2 ng digoxigenin-labeled GAP-LOCK first probes (see below) in 100 µl hybridization buffer (62.5 mM Na2HPO4, 0.94 M NaCl, 94 mM citric acid, 10 mM MgCl2, 0.125% Tween-20, 0.0625% BSA, 15 mM NaN3, pH 6.5) (final concentration equal to 5.5x SSC). Hybridization at 42°C for 2 hrs was followed by washing with wash buffer. Anti-digoxigenin aequorin conjugate (5 ng in 100 µl) was added for 30 min followed by detection measuring enhanced flash (469 nm) on a luminometer using a calcium trigger solution (50 mM Tris, 10 mM calcium acetate, pH 7.5, 15 mM NaN3). The limit of detection for the luminometer was previously determined empirically at 2.1×10^{-17} moles.

Modified Spacer Primer and Reverse Primer to construct GAP-LOCK First Probe for MTB by PCR:

A. First Probe Oligomer: (SEQ ID NO: 51,52)

| target region C9 spacers M13 forward primer |

5'ACCACAAGACATGCATCCCG-99-CCAGGGTTTCCCAGTCACGAC

B. Second M13 R Primer: (SEQ ID NO: 53) 5' GAGCGGATAACAATTTCACACAGG

Fabrication: Oligomers A and B were synthesized. Using Oligomers A and B as primers, a PCR reaction was run using an arbitrary M13 DNA template (300 bp in length) to produce a long double-stranded reporter containing digoxigenin (1:3 ratio) with a single-stranded probe on the 5' end.

Application Steps: The probe was hybridized to denatured, biotinylated target DNA, captured on the surface of the wells. Anti-dig aequorin (Sealite Sciences, Inc. Norcross, GA) was added; the microtiter plate was put in a luminometer; calcium was added to trigger

aequorin; and detection levels were recorded. The results in Figure 21B show highly sensitive detection.

Example 10

GAP-LOCK Probe Method and TINKER-TAG Method with Aequorin Bioluminescence:

I. Probe Components: (targeting sense strand of MTB 16s RNA sequences)

A. 5' GAP-LOCK Capture Probe Subunit: (SEQ ID NO: 54)
| upstream target region spacers link to 3' end of C (capture subunit)|
5' Phosphate--GGTCCTATCCGGTATTAGA-TTT-CTAGGGTTACCATA

B. 3' GAP-LOCK Reporter Probe Subunit: (SEQ ID NO: 55)
| link to TINKER-TAGs spacers downstream target region |
5' GTAGCCTAGCTACCCCTAGGTCTAG-TTT-ACCACAAGACATGCATCCCGT

Capture Subunit: (SEQ ID NO: 56)
C. 5' Biotin--TATGGTAACCCTAG

Using methods described in Examples 1-9, synthesize oligonucleotides A, B and C. Anneal and crosslink A and C with free psoralen plus long wave UV to make Capture Probe Subunit.

I. TINKER-TAG Reporter Components:

D. Proximal Subunit: (SEQ ID NO: 57)
| 1st link to probe - overlap - side link to Label Subunit |
5'CTAGACCTAGGGGTAGCTAGGCTAC-ATACGATACTAG-GGCATAACATAGGCTTACCA

E. Distal Subunit: (SEQ ID NO: 58)
| 1st link to next proximal subunit - overlap - side link to Label Subunit |
5'GTAGCCTAGCTACCCCTAGGTCTAG-CTAGTATCGTAT-GGCATAACATAGGCTTACCA

F. Label Subunit: (SEQ ID NO: 59)
5' Aequorin Label - TGGTAAGCCTATGTTATGCC

G. Terminator: (SEQ ID NO: 60) 5' CTAGACCTAGGGGTAGCTAGGCTAC

Using methods described in Examples 1-9, synthesize oligonucleotides D, E, F and G. Hybridize and crosslink multiple D and E subunits together to form linear TINKER-TAG chains, length limited by the addition of a small ratio (e.g. 1:6) of Terminators (G subunit). See Figure 6. The GAP-LOCK Reporter Probe Subunits can be added and crosslinked to the TINKER TAG units at this stage. See Figure 16. The Label Subunits (F) conjugated with aequorin will not be added to the linker side arms of the TINKER TAGs at this step.

Using methods described in Examples 1-9, mix and hybridize denatured target DNA or RNA with GAP-LOCK Reporter Probe Subunit and Capture Probe Subunit with Biotin moiety attached. The TINKER TAG units may be preattached to the Reporter Probe Subunit. Ligate the Probe Subunits together and transfer sample to capture-ready mictrotiter plates coated with strepavidin. Denature and wash to remove unligated Reporter Probe Subunits and released target strands. Add Aequorin Label Subunits to link to side arms of TINKER TAGs bound to captured probe complex. Wash to remove unlinked Aequorin Label Subunits. Transfer plate to luminometer and record detection by triggering aequorin flash with calcium stimulation.

The method should detect and disctinguish 16s MTB RNA sequences (common TB) from Mycobacterium avium sequences (the AIDS prevalent TB) which differ from the MTB target sequences by 4 bases in the nucleotides immediately adjacent to the GAP-LOCK probe ligation site that is employed.

Example 11

GAP-LOCK First Probe Method with FISH detection:

GAP-LOCK First Probes for FISH detection were created with digoxigenins (dig-11-dUTP) incorporated in the attached reporter tail (1:3 substitution ratio for thymidine bases) to target a chromosome 12 specific site adjacent to the centromere which has multiple tandem repeat elements. Because unligated GAP-LOCK First Probes with long reporter tails are labile, probes for FISH detection were constructed with a short reporter tail of 300 bp and a large 30 bp probe "footprint" of target specific sequences. BLAST analysis of the 30 mer sequences against the GenBank libraries (GenBank, EMBL, DDBJ, PDB) show two published fragments (171 and 685 bp) with single sites with a 100% match, and when

including another two published fragments (340 and 1359 bp), a total of five other sites match the First Probe sequences with homologies of 82% to 96%. The probe was hybridized to the chromosome target at 37°C for 14 hours, washed and treated with anti-digoxigenin FITC stain and propidium iodide counterstain by standard methods as described above.

The staining with this Chromosome 12 GAP-LOCK First Probe was very distinctive as compared to standard FISH probes. Virtually every metaphase cell showed clear sharp dots or bands on the correct chromosome region and nearly all interphase cells showed the expected 2 dot or 4 dot labeling configurations. These results were repeated three times.

Using the same methods, additional GAP-LOCK First Probes were prepared for FISH with a similar configuration (30 bp target end, 300 bp reporter tail) to detect an Alu consensus sequence. These results showed good FISH labeling of chromosome bands which correspond to the R-band-like patterning of active genes.

Example 12

WRAP-PROBE and COLOR-TAG Methods for FISH detection:

The following methods apply to the WRAP-PROBE and COLOR-TAG methods in the FISH format using human chromosome and nuclei preparations to detect and map the ABR gene and the 15-LO-1 gene on the short arm of Chromosome 17 with respect to the microsatellite marker D17S379 on the same chromosome. A control probe of similar design is also provided to detect and map the repetitive subcentromeric site on Chromosome 12 which was previously mapped with the GAP-LOCK First Probe method described in Example 11 above. The oligonucleotides described include all components needed for preparing WRAP-PROBEs and COLOR-LINKER chains for the four target sites, as well as all components required to make four different COLOR-TAG reporters as needed to provide mixed three-color detection. However, to distinguish these sites, only two color reporters need be made, and so this example will only use RED and GREEN COLOR-TAGs. Red labeling is provided by anti-biotin rhodamine fluorescence. Green labeling is provided by anti-digoxygenin FITC or fluorescein. To simplify this example, the ABR probe is designed to only attract RED COLOR-TAGs, the 15-LO-1 probe only attracts GREEN COLOR-TAGs, and both the D17S379 probe and the Chromosome 12 site probe attracts a mix of half RED

and half GREEN COLOR-TAGs. In FISH detection, the green fluorescent compound, FITC or fluorescein, actually gives a yellow green color, and the mixed color probes will give a yellow orange color. These probes will be applied to microscope slides containing human chromosomes and nuclei that are counterstained blue with DAPI since the resulting three visual probe colors, red, yellow and green, are known to contrast well with this background.

WRAP-PROBE Target Specific Oligomers: (SEQ ID NO: 61)
A 1: CHR-17: ABR gene:
5' CTAGCGTACACCTA-TTT-GAATCCCTTGAAGCCTGAGA-TTT-GTAGATAGGTAG

A 2: CHR-17: 15-LO-1 gene: (SEQ ID NO: 62)
5' CTAGCGTACACCTA-TTT-CAGACAACAGGGAGGCAGCGGCTTTTA-TTT-
GTAGATAGGTAG

A 3: CHR-17: D17S379 microsatellite marker: (SEQ ID NO: 63)
5' CTAGCGTACACCTA-TTT-ACAGTATGTTTTAGTGAATGAATAGATC
-TTT-GTAGATAGGTAG
A 4: CHR-12 subcentromere site: (SEQ ID NO: 64)
5' CTAGCGTACACCTA-TTT-AAAGTGATTGAAATCTCCAACTGGAAACTG
-TTT-GTAGATAGGTAG
WRAP-PROBE generic Reversing Linker: (SEQ ID NO: 65)
B: 5' TAGGTGTACGCTAG--GTAGATAGGTAG

WRAP-PROBE generic COLOR-LINKER Chain Oligomers:
C: Generic Overlap Oligomer: (SEQ ID NO: 66)
5' CTAGTTAGCCTAC--GTAGATAGGTAG

D: RED Reporter Acceptor Oligomers: (SEQ ID NO: 67)
5' GTAGGCTAACCCTAT-CTACGATACGATAGGGCCTAAGAGTAG-
CGACTACCTATCTAC

E: GREEN Reporter Acceptor Oligomers: (SEQ ID NO: 68)
5' GTAGGCTAACCCTAT-GCCTAGACCTAGGGGTAGCTAGGCTAC-
CGACTACCTATCTAC

F: BLUE Reporter Acceptor Oligomers: (SEQ ID NO: 69)
5' GTAGGCTAACCCTAT-CGTAGACCTAGCACGCTACGTACTAGG-
CGACTACCTATCTAC

G: Generic Chain Terminator: (SEQ ID NO: 70)
5' ATAGGGTTAGCCTAC

RED GENE-TAG Oligonucleotides: (SEQ ID NO: 71,72)

H: Proximal Spacer Oligomer with two 9 carbon spacers: (creates proximal linker)
5' CTACTCTTAGGCCCTATCGTATCGTAG--99--CCAGGGTTTTCCCAGTCACGAC

I: Distal Spacer Oligomer with two 9 carbon spacers: (creates distal linker) (SEQ ID NO: 73,74)
5' CTACGATACGATAGGGCCTAAGAGTAG--99--
GAGCGGATAACAATTTCACACAGG

J: RED Terminator: (SEQ ID NO: 75)
5' CTACTCTTAGGCCCTATCGTATCGTAG

GREEN GENE-TAG Oligonucleotides: (SEQ ID NO: 76,77)
K: Proximal Spacer Oligomer with two 9 carbon spacers: (creates proximal linker)
5' GTAGCCTAGCTACCCCTAGGTCTAGGC--99--CCAGGGTTTTCCCAGTCACGAC

L: Distal Spacer Oligomer with two 9 carbon spacers: (creates distal linker) (SEQ ID NO: 78,79)
5' GCCTAGACCTAGGGGTAGCTAGGCTAC--99--
GAGCGGATAACAATTTCACACAGG

M: GREEN Terminator: (SEQ ID NO: 80)
5' GTAGCCTAGCTACCCCTAGGTCTAGGC

BLUE GENE-TAG Oligonucleotides: (SEQ ID NO: 81,82)
N: Proximal Spacer Oligomer with two 9 carbon spacers: (creates proximal linker)
5' CCTAGTACGTAGCGTGCTAGGTCTACG--99--CCAGGGTTTTCCCAGTCACGAC

O: Distal Spacer Oligomer with two 9 carbon spacers: (creates distal linker) (SEQ ID NO: 83,84)
5' CGTAGACCTAGCACGCTACGTACTAGG--99--
GAGCGGATAACAATTTCACACAGG

P: BLUE Terminator: (SEQ. ID NO. 85):
5' CCTAGTACGTAGCGTGCTAGGTCTACG

1. Probe assembly: Using methods described in Examples 1, 2-4 and 11, synthesize above oligonucleotides A to P and purify. Construct WRAP-PROBEs separately by mixing equimolar portions of Target Specific Oligomer and Reversing Linker Oligomers in separate tubes in PCR buffer at 42°C for 10 min as follows: For ABR gene probe: 1:1 ratio ABR Target Specific Oligomer (A1) with Reversing Linker (B) For 15-LO-1 gene probe: 1:1 ratio 15-LO-1 Target Specific Oligomer (A2) with Reversing Linker (B)For D17S379 marker

probe: 1:1 ratio D17S379 Target Specific Oligomer (A3) with Reversing Linker (B) For CHR-12 site probe: 1:1 ratio CHR-12 Target Specific Oligomer (A4) with Reversing Linker (B)

Construct Chain subunits separately by mixing color specific Reporter Acceptor Oligomers with generic Chain Overlap Oligomers and generic Chain Terminators in molar proportions as follows:

Chain specificity: Molar Ratio: Component Name:
For ABR - RED only: a. 14: Chain Overlap Oligomers (C)
b. 16: RED Reporter
Acceptor Oligomers (D)
c. 2: Chain Terminators (G)

Chain specificity: Molar Ratio: Component Name:
For 15-LO-1 - GREEN only: a. 14: Chain Overlap Oligomers (C)
b. 16: GREEN Reporter
Acceptor Oligomers (D)
c. 2: Chain Terminators (G)

Chain specificity: Molar Ratio: Component Name:
For D17S379- 1:1 RED/GREEN: a. 14: Chain Overlap Oligomers (C)
b. 8: RED Reporter
Acceptor Oligomers (D)
c. 8: GREEN Reporter
Acceptor Oligomers (D)
d. 2: Chain Terminators (G)

Chain specificity: Molar Ratio: Component Name:
For CHR-12 - 1:1 RED/GREEN: a. 14: Chain Overlap Oligomers (C)
b. 8: RED Reporter
Acceptor Oligomers (D)
c. 8: GREEN Reporter
Acceptor Oligomers (D)
d. 2: Chain Terminators (G)

Hybridize Chain Units in three separate tubes in PCR buffer at 42°C for 20 min. To each tube of the three different Chain Units, add an equal volume proportion of the corresponding pre-hybridized WRAP-PROBE to join probe complex and branch arm complex together. Then crosslink all components together by adding 3 ug/ml psoralen (4,5',8-trimethylpsoralen) to each tube for 10 min. Treat with 15 W blacklite lamp (360 nm) at 1 inch for 15 min. Use clear thin wall PCR tubes. Purify the three probes on Microcon 30 microfuge columns.

These ratios should give one probe molecule per every two Chain subunits. Each WRAP-PROBE should therefore have approximately eight GENE-TAG acceptor sites on each Chain extending from the terminal ends of the probe, for a total of about sixteen such sites per probe. The total length of the probe with two Chains should be less that 900 bases.

2. RED Reporter Amplification and Hapten Labeling: Generate RED reporters by PCR using 2 μl RED Forward Primer with spacers, 2 μl RED Reverse Primer, 2 μl M13 template (300 bp), and incorporate biotin-16-dUTP in ratio: 1 biotinylated base per 3 thymidine bases. Purify reporters with Microcon 100 microfuge columns.

3. GREEN Reporter Amplification and Hapten Labeling: Generate GREEN reporters by PCR using 2 μl GREEN Forward Primer with spacers, 2 μl GREEN Reverse Primer, 2 μl M13 template (300 bp), and incorporate digoxyigenin-11-dUTP in ratio: 1 biotinylated base per 3 thymidine bases. Purify reporters with Microcon 100 microfuge columns.

4. GENE-TAG Chain Assembly: Pre-mix and hybridize in two separate tubes:
RED COLOR-TAGs with RED Terminator molecules, GREEN COLOR-TAGs with GREEN Terminator molecules, in PCR buffer at 47oC for 20 min, using different molar ratios as needed for signaling. Start with 3:1 ratio; progress to 10:1 ratio if required.

5. Hybridize Probe/Chain units to denatured, dehydrated target DNA at 37°C for 2 to 6 hrs.

a. ABR Probe/Chain unit w/ RED reporter acceptors
b. 15-LO-1 Probe/Chain unit w/ GREEN reporter acceptors

c. D17S34 Probe/Chain unit w/ 1:1 RED/GREEN reporter acceptors

d. CHR-12 site Probe/Chain unit w/ 1:1 RED/GREEN reporter acceptors

6. Rinse with hot hybridization buffer (42-47°C) containing equal amounts of RED and GREEN reporter chains. (Alternatively, add RED and GREEN COLOR-TAGs and RED and GREEN Terminators in molar proportions as described in step 4 above.) Continue 42°C hybridization for 4 to 8 hours.

7. Rinse and add anti-digoxygenin FITC and anti-biotin Rhodamine or Texas Red, DAPI counterstain.

8. Fluorescent microscope analysis and photography.

Other Experimental Protocols relating to FISH and COLOR-TAG :

Lymphocyte cultures are grown by standard cytogenetic methods using PHA stimulation and colchicine mitotic arrest to harvest metaphase cells. Cells are swollen by KCl hypotonic and fixed with 3:1 methanol/acetic acid.

The hybridization and post hybridization protocols are based in part on Reid et al. [Proc Natl Acad Sci USA Vol. 89, pp. 1388-1392 (1992)], Wu et al. [Genomics, Vol. 17, pp. 163-70 (1993)] and the modifications of Matera and Ward [Hum. Mol. Genetics, Vol. 1, pp. 535-539 (1992)] that are specific for oligonucleotide based FISH probes. Slides with metaphase chromosomes or interphase nuclei are denatured in 70% formamide in 2xSSC for 2 min. at 72o C, dehydrated with 2 min. rinses in 70, 90 and 100% ice cold ethanol, and air dried. For each slide, 10 to 25 microliters of hybridization mixture is used containing 20 ng of the WRAP-PROBEs, 20 to 40% formamide, 5 to 10% dextran sulfate, 2xSSC, 0.1% SDS, 5xDenhart's solution, 1xBackground Quencher (MRC) and 40 mM sodium phosphate. In situ hybridization is maintained at 37o to 42o C in a humidified chamber. Probe hybridization is tested in the range of 2 to 6 hours. COLOR-TAG reporter hybridization is tested at 4 to 8 hours, but up to 18 hours if needed. Adjustments in formamide concentration and temperature are essential for optimizing FISH. Denaturing the double-stranded COLOR-TAG reporters prior to or during hybridization is avoided.

After hybridization, the slides are washed in 20 to 45% formamide/2xSSC for 10 min at 37 to 42o C, followed by 0.1xSSC for 15 min at 55o C. Digoxygenin or biotin labeled preps are incubated at 37oC for 5 min. with either 60 μl of anti-dig conjugated FITC or avidin conjugated rhodamine. Slides are then washed for 3 min. each in 2xSSC with 0.1% Triton X-100 at room temperature. Slides are generally counterstained with 18 μl of either propidium iodide (orange-red) or DAPI (pale blue) with antifade. FISH stained cells are detected by fluorescent microscopy using a triple bandpass filter and photographed with Kodak Elite 400 color film. Image processing is conducted with a Cytovision Imaging System optimized for FISH (Applied Imaging).

After COLOR-TAG based FISH analysis in this example, interphase cells were observed with spectrally confirmed labeling patterns indicative of fluorescent detection of the three color signals, red only, green only, and red/green mixed 50/50, and with appropriate localizations for the four probes. See Figure 13. Thus, the WRAP-PROBEs and chained COLOR-TAGs were adequately conjoined to produce considerably more signaling than has been achieved with oligonucleotide based probes. Metaphase detection would be required to demonstrate the mappling potential of this technology.

Example 13

Duo-Layered GENE-TAG Method

In order to apply chains of GENE-TAG reporters in a more controlled manner, two types of GENE-TAGs were created that could stack on one another end to end but that would not form chains in solution when kept separately. See Figure 7A. These alternating GENE-TAGs were constructed by using the modified spacer primers that were previously employed in Example 12 above to create GREEN and RED COLOR-TAGs for the COLOR-TAG system. In this case, however, the PCR primer pairs for the reporter template were switched by combining the Proximal Spacer Oligomer of the RED COLOR-TAGs and the Distal Spacer Oligomer of the GREEN COLOR-TAG units to make Type I GENE-TAGs, and conversely the Proximal Spacer Oligomer of the GREEN COLOR-TAGs was combined with the Distal Spacer Oligomer of the RED COLOR-TAG units to make Type II GENE-TAGs. Thus if a WRAP-PROBE or other probe provided linkers for joining RED COLOR-TAGs,

Type I COLOR-TAGs could be applied. However, since the distal end of this Type I reporter has GREEN linker sequences, only one such COLOR-TAG would bind to each probe forming a single layer of GENE-TAGs on a group of bound probes. Then following this step, with a wash step in between, a Type II GENE-TAG could be similarly applied, to form a second layer, since this GENE-TAG would bind to the distal termini of the prior Type I layer. Thus, alternating layers of Type I and Type II could be applied successively. See Figure 7B.

Type I Duo GENE-TAG Oligonucleotides:

A: Proximal Spacer Oligomer with two 9 carbon spacers: (creates proximal linker) (SEQ ID NO. 86, 87)
5' CTACTCTTAGGCCCTATCGTATCGTAG--99--CCAGGGTTTTCCCAGTCACGAC

B: Distal Spacer Oligomer with two 9 carbon spacers: (creates distal linker)(SEQ ID NO. 88, 89)
5' GCCTAGACCTAGGGGTAGCTAGGCTAC--99--
GAGCGGATAACAATTTCACACAGG

Type II Duo GENE-TAG Oligonucleotides: (SEQ ID NO. 90, 91)
C: Proximal Spacer Oligomer with two 9 carbon spacers: (creates proximal linker)
5' GTAGCCTAGCTACCCCTAGGTCTAGGC--99--CCAGGGTTTTCCCAGTCACGAC

D: Distal Spacer Oligomer with two 9 carbon spacers: (creates distal linker) (SEQ ID NO. 92, 93)
5' CTACGATACGATAGGGCCTAAGAGTAG--99--
GAGCGGATAACAATTTCACACAGG

Duo GENE-TAG fabrication: Using methods described in Examples 1, 2, 4, 6, 7 and 12, synthesize oligonucleotides A, B, C and D. Oligomer set A and B and oligomer set C and D serve as primers in two separate PCR reactions using a M13 DNA template 600 bp long to create Type I and Type II GENE-TAGs. Radioactive P32 is incorporated in 1:3 thymidine bases during PCR.

Application Steps: Using methods described in Examples 1, 2, 4, 6 and 7, mix and hybridize denatured target DNA or RNA with WRAP-PROBEs targeted to a membrane bound substrate. Add Type I GENE-TAG reporters to bind via complementary linkers to bound probe. Remove solution and wash briefly to remove unbound GENE-TAGs. Removed solution with Type I GENE-TAGs may be retained and reapplied at a latter step. Add Type II GENE-TAG reporters to bind via complementary linkers to bound Type I GENE-TAGs.

Remove solution and wash briefly to remove unbound GENE-TAGs. Repeat above steps with Type I followed by Type II in succession as needed. In current practice only one to three layers were applied. Radioactive labeling is then read with a phosphoimager for 2 to 4 hours or with x-ray film exposure for 6 to 18 hours. This layered application of Type I and Type II GENE-TAGs was employed and compared to the use of standard GENE-TAGs joined in chains. The spacer primer set to make RED GENE-TAGs was employed to make P32 labeled standard GENE-TAGs and these products were mixed with a terminator oligomer on a 3:1 basis to produce chains averaging three GENE-TAGs long. In parallel, the Type I and Type II GENE-TAGs were made at the same time and they were labeled by the same protocol as described above.

Nylon membranes were prepared with slot blot depositions of 10^{n} copies of the target DNA, 15-LO-1, a control DNA, TNF a, and a second control sample such as sterile water. WRAP probes containing a 257 bp sequence of 15-LO-1 cDNA were prepared with a biotinylated primer containing overlap linker sequences and with oligonucleotide adapters containing a terminal GENE-TAG Linker sequence corresponding to that of the RED COLOR-TAGs or to the proximal linker of the Type I GENE-TAGs.

The probe was hybridized overnight at 48 degrees C., washed and then either the 3:1 chain of standard GENE-TAGs or a Type I GENE-TAG was applied to each sample in a roller hybridization apparatus at 42 degrees C. The chain of standard GENE-TAGs was hybridized to the probe complex for 6 hours and detection was accomplished with x-ray film. See Figure 8A. With the alternate layering protocol, three samples were prepared for each concentration and treated separately. In the first sample, the Type I GENE-TAGs were hybridized for 2 hours, then washed and layered with Type II GENE-TAGs for another two hours, followed by a third treatment for two hours with Type I GENE-TAGs. In the second sample, only two such layers were applied, and in the third sample, only one such layer was applied. Thus, all samples, including those treated with a 3:1 chain of standard GENE-TAGs, were then removed and applied at the same time to x-ray film for comparative detection. See Figure 8B.

The results of several such experiments indicate that layered GENE-TAGs provide more efficient and sensitive detection than standard GENE-TAGs joined in a chain and applied together.

Example 14

Double Duo GENE-TAG Method

In order to provide branching rather than linear arrays of GENE-TAG reporters, the Type I and Type II GENE-TAG reporters of Example 13 were additionally created with double distal linkers in tandem so that signaling could be doubled with each layer that is applied. See Figure 9. This method applies the Type I and the Type II GENE-TAGs in sequential manner using the same methods and procedures of Example 13 except that the following modified spacer oligomers are employed in manufacturing Type I and Type II GENE-TAGs with double distal linkers.

Type I Double Duo GENE-TAG Oligonucleotides: (SEQ ID NO. 94,95)
A: Proximal Spacer Oligomer with two 9 carbon spacers: (creates proximal linker)
5' CTACTCTTAGGCCCTATCGTATCGTAG--99--CCAGGGTTTTCCCAGTCACGAC

B: Double Distal Spacer Oligomer with two 9 carbon spacers: (creates double distal linker) (SEQ ID NO. 96,97,98)
5'
GCCTAGACCTAGGGGTAGCTAGGCTAC--
99GCTAGACCTAGGGGTAGCTAGGCTAC--99--GAGCGGATAA
CAATTTCACACAGG

Type II Double Duo GENE-TAG Oligonucleotides: (SEQ ID NO. 99,100)
C: Proximal Spacer Oligomer with two 9 carbon spacers: (creates proximal linker)
5' GTAGCCTAGCTACCCCTAGGTCTAGGC--99--CCAGGGTTTTCCCAGTCACGAC

D: Double Distal Spacer Oligomer with two 9 carbon spacers: (creates double distal linker) (SEQ ID NO. 101,102,103)
5'
CTACGATACGATAGGGCCTAAGAGTAG--99--
CTACGATACGATAGGGCCTAAGAGTAG--99--GAGCGGATAA
CAATTTCACACAGG

Example 15

Two-Part GENE-TAG Multi-LINKER

In order to provide branching linkers for the binding of multiple GENE-TAGs to a WRAP-PROBE, GAP-LOCK probe or other probes affixed with complementary linkers, a set of Two Part Multi-LINKERs were created. These multi-linkers were designed so that both the first linker to be joined to the probe and the multiple terminal distal linkers to be joined to the GENE-TAGs have sufficient length and sequence exclusivity to join these components durably and reliably in an in situ situation, that is where the probe and target have been hybridized together in a detection format and diverse non-target nucleotide sequences may be available in the target sample. In addition, the first and distal linkers of the composite Two-Part Multi-LINKER have the capacity to be crosslinked to either the probe or to GENE-TAGs either before application or afterwards.

Two-Part GENE-TAG Multi-LINKER

First Component: One to Four Multi-Linker (SEQ ID NO. 104,105,106,107,108)
5'
CTACTCTTAGGCCCTATCGTATCGTAG-9-GTAATAGCGTAC-9-GTAATAGCGTAC-9-GTAATAGCGTAC-9-G
TAATAGCGTAC

Second Component: One to Two Multi-Linker (SEQ ID NO. 109,110,111)
5' GCCTAGACCTAGGGGTAGCTAGGCTAC-99-
GCCTAGACCTAGGGGTAGCTAGGCTAC-99-GTACGCTATTAC

The Multi-LINKER components are hybridized together in vitro with a molar ratio of the second components that is at least four times the molar quantity of the first component using the methods described in Example 12 above. If the probe and Multi-LINKER are to be crosslinked together, these components are mixed together under the same hybridizing conditions and the crosslinking of all components with psoralen and UV is performed in a single step rather than sequentially since a second hit with UV may break the covalent bonds established previously.

Example 16

Three-Part Oligo-TAG Multi-LINKER

In order to provide branching Multi-Linkers for the binding of multiple short labeled oligonucleotides, called Oligo-TAGs, to a WRAP-PROBE, GAP-LOCK probe or other probes affixed with complementary linkers, a set of Three-Part Multi-LINKERs were manufactured. These multi-linkers were designed so that the first linker to be joined to the probe has sufficient length and sequence exclusivity to join these components durably and reliably in an in situ situation, that is where the probe and target have been hybridized together in a detection format and diverse non-target nucleotides may be available in the target sample. In addition, the first linker of the composite Three-Part Multi-LINKER may be crosslinked to the linker of the probe.

These Three-Part Multi-LINKERs were also designed in two sets so that comparative labeling could be employed where one probe would have the capacity to bind or would be prebound to the first multi-linker set and where a second probe would have the capacity to bind or would be prebound to the second multi-linker set. This particular two set example was designed and made to bind labeled oligonucleotides with the fluorescent compounds Cy3 and Cy5 which are commonly employed in comparative hybridizations with DNA chip technologies and FISH protocols. The Cy3 and Cy5 components are commercially available in phosphoramidite form allowing them to be easily added to the 5' end of an oligonucleotide duringsynthesis using common commercial synthesizers.

Three-Part Oligo-TAG Multi-LINKERs

Red Set for Cy5 labeled Oligo-TAGs (SEQ ID NO. 112,113,114,115,116)
One to Four First Linker
5'
CTACTCTTAGGCCCTATCGTATCGTAG-9-GTAATAGCGTAC-9-GTAATAGCGTAC-9-GTAATAGCGTAC-9-G
TAATAGCGTAC

One to Four Second Linker (SEQ ID NO. 117,118,119,120,121)
5' CTAGGTAGCTAG-9-CTAGGTAGCTAG-9-CTAGGTAGCTAG-9-CTAGGTAGCTAG-9-GTACGCTATTAC

One to Four Third Linker (SEQ ID NO. 122,123,124,125,126)
5' CTAGCTACCTAG-99-GTACGTAACTAG-99-GTACGTAACTAG-99-GTACGTAACTAG-99-GTACGTAACTAG

Red Set Oligo-TAG with Cy5 (SEQ ID NO. 127)

5' cy5--CTAGTTACGTAC

Green Set for Cy3 labeled Oligo-TAGs (SEQ ID NO. 128,129,130,131,132)
One to Four First Linker
5'
GCCTAGACCTAGGGGTAGCTAGGCTAC-9-CTACCTATCTAC-9-CTACCTATCTAC-9-CTACCTATCTAC-9-C
TACCTATCTAC

One to Four Second Linker (SEQ ID NO. 133,134,135,136,137)
5' CTAGGTAGCTAG-9-CTAGGTAGCTAG-9-CTAGGTAGCTAG-9-CTAGGTAGCTAG-9-GTACGCTATTAC

One to Four Third Linker (SEQ ID NO. 138,139,140,141, 142)
5' CTAGCTACCTAG-99-CTATCTAGTACG-99-CTATCTAGTACG-99-
CTATCTAGTACG-99-CTATCTAGTACG

Green Set Oligo-TAG with Cy3 (SEQ ID NO. 147)
5' cy3--CGTACTAGATAG

The Multi-LINKER components of each set are hybridized together in vitro with a molar ratio of the second components that is at least four times the molar quantity of the first component, and with the molar ratio of the third components that is at least four times the molar quantity of the second component, using the methods described in Example 12 and 15 above. If the probe and Multi-LINKER are to be crosslinked together, these components are mixed together under the same hybridizing conditions, and the crosslinking of all components with psoralen and UV is performed in a single step rather than sequentially as described in Examples 12 and 16 above.

Example 17

Double-WRAP FISH PROBEs

In order to map genes more reliably by FISH, two WRAP-PROBEs are made to each target sequence one being complementary to the sense strand and the other being complementary to the antisense strand. The two WRAP-PROBEs are made with different linker sequences as described in Examples 8 above for DOUBLE-LOCK probes in order to bind different reporters such as the RED and GREEN GENE-TAGs of Example 12 above,

and they are applied and detected in the same manner using the FISH protocols described in Example 12 . The example is for a pair of Double-WRAP FISH PROBEs for the promoter region of the 15-LO-1 gene on Chromosome 17.

WRAP-PROBEs: CHR-17: 15-LO-1 gene (SEQ ID NO. 143)
Sense Target Oligomer:
1. 5'
CTACGATACGATAGGGGTAAGAGTAG--TTT--
CAGACAACAGGGAGGCAGCGGCTTTTA--TTT--GTAGATAGG
TAG

Sense Overlap Oligomer (SEQ ID NO. 144)
2. 5' CTACGATACGATAGGGGTAAGAGTAG--TTT--CTACCTATCTAC

Anti-Sense Target Oligomer: (SEQ ID NO. 145)
3. 5'
GCCTAGACCTAGGGGTAGCTAGGCTAC--TTT--
TAAAAGCCGCTGCCTCCCTGTTGTCTG-TTT--GTAGATAGG
TAG

Anti-Sense Overlap Oligomer (SEQ ID NO. 146)
4. 5' GCCTAGACCTAGGGGTAGCTAGGCTAC--TTT--CTACCTATCTAC

Hybridize and crosslink components 1 and 2 together in vitro and do the same with components 2 and 3 in a separate tube using the methods described in Example 12. Hybridize probes separately or simultaneously to the target sample, and wash and hybridize RED and GREEN GENE-TAGs to the probe and target complexes. The RED and GREEN GENE-TAGs may be applied in linear chains or branching chains using double distal linkers as described in Example 14 above. The protocols of Examples 8 and 12 are used to detect the two colored reporters separately and thus confirm a valid target detection.

The present invention relates generally to a modular method of integrating different gene probe methods and reporter methods for biological research and diagnostics by designing a set of nucleic acid probe compositions and a set of related reporter compositions that can be interchanged and joined together, either directly or indirectly. The present invention consists in part of the design and employment of common sets of artificial complementary nucleotide linker sequences that can join, align or multiplex different probe and reporter units together in a functional and versatile manner. The present invention

consists in part of the design and employment of an additional set of interconnecting polynucleotide compositions, known as multilinkers, that similarly join, align, amplify or bring into functional association the aforesaid probe and reporter compositions by virtue of designing and making said multilinkers with sets of nucleotide sequences that allow partial or complete self assembly and that correspond in whole or in part to the common linker sets of the probe and reporter compositions of the present invention. The present invention also consists in part of the future probe and reporter compositions and methods that may herewith be created by virtue of employing the modular design principles of the present invention, or by employing the same or similar linker sets and multilinker compositions that are herein embodied.

While the foregoing specification teaches the principles of the present invention, with examples provided for the purpose of illustration, it will be understood that the practice of the invention encompasses all of the usual variations, adaptations, and modifications, as come within the scope of the following claims and its equivalents.